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Phytotoxic Compounds produced by *Fusarium equiseti*

P. W. BRIAN, A. W. DAWKINS, JOHN FREDERICK GROVE, H. G. HEMMING, D. LOWE, AND G. L. F. NORRIS

Akers Research Laboratories, Imperial Chemical Industries Ltd., Welwyn, Herts.

With one Figure in the Text

Received 6 May 1960

SUMMARY

Culture filtrates of several strains of *Fusarium equiseti* (Corda) Sacc. were found to be highly phytotoxic. Several phytotoxic substances were isolated; the most important of these was a colourless crystalline compound, diacetoxyscirpenol, m.p. 161–2°, $C_{19}H_{26}O_7$. Though highly phytotoxic in many respects, it markedly stimulates elongation of cress roots at 0·01–0·5 μg./ml. This is not an anti-auxin effect. Diacetoxyscirpenol is at most only slightly inhibitory to fungi and bacteria, but is highly toxic to rats. Two minor metabolic products, m.p. 135–6° and 185–8° respectively, are also described. They, too, were highly phytotoxic, though their spectra of activity were somewhat different from that of diacetoxyscirpenol. They also were highly toxic to rats. Production of these substances is probably not important in the aetiology of disease symptoms in plants parasitized by *F. equiseti*, but may be significant in cases of toxicosis of animals fed with grain infected by Fusaria. Scirpentriol, a hydrolysis product of diacetoxyscirpenol, is much less phytotoxic but of equal toxicity to rats.

INTRODUCTION

In continuation of work on production of gibberellic acid by strains of *Fusarium moniliforme* (Borrow *et al.*, 1955) we have extended our interest to other *Fusarium* spp. No evidence has been obtained of gibberellin production by species other than *F. moniliforme*, but several species have been found to produce phytotoxic metabolites. This paper describes the properties of such substances produced by *Fusarium equiseti* (Corda) Sacc.

MATERIALS AND METHODS

Fungi. The organisms used are listed in the text elsewhere.

Culture methods. In preliminary experiments, fungi were grown on 25-ml. lots of medium in 100-ml. conical flasks. The media used were Czapek–Dox (Brian, Curtis, and Hemming, 1946) and a glucose : ammonium nitrate (GAN) medium at the following composition: glucose 50·0 g., NH_4NO_3 2·4 g., KH_2PO_4 10·0 g., $MgSO_4.7H_2O$ 2·0 g., distilled water 1 l. To both media a minor element concentrate (Brian, Curtis, and Hemming, 1946) was added at a rate of 2 ml. per litre of medium. In subsequent work with chosen fungi the process was scaled-up by using larger culture vessels. All cultures were grown at 25° C.

Pea section bioassay. This method involves measurement of extension of 5-mm. sections cut from upper internodes of 'Meteor' pea seedlings, the test

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Pea section bioassay. This method involves measurement of extension of 5-mm. sections cut from upper internodes of 'Meteor' pea seedlings, the test

being carried out in light. The normal basal medium used contains 10 μg./ml. indolylacetic acid and 1 per cent. sucrose in pH 6·1 phosphate buffer (Brian and Hemming, 1958).

Cress root growth test. This has been described by Brian, Hemming, and Radley (1955).

Bean leaf-disk bioassay. Disks 5 mm. in diameter are cut from leaves of *Phaseolus vulgaris* var. 'Masterpiece', using light-grown plants. The disks are floated on a medium of 1 per cent. sucrose and 0·1 μg./ml. indolylacetic acid in pH 6·1 phosphate buffer at 15° C. in high-intensity light. Diameters of disks are measured after 48 hr.

PRELIMINARY SURVEY OF FUSARIA

The organisms examined were obtained either from the Commonwealth Mycological Institute, Kew, or from the Centraalbureau voor Schimmelcultures, Baarn, Holland. They are listed below by the names under which they were received: *Fusarium anguioides* Sherb., *F. angustum* Sherb., *F. annulatum* Bugnicourt, *F. apii* Nelson and Sherb., *F. avenaceum* (Fr.) Sacc. (3 strains), *F. bostrycoides* Wollenw. and Reinking, *F. bucharicum* Jacz., *F. caucasicum* Let., *F. cavispermum* Corda, *F. citriforme* Jamaleinen, *F. coeruleum* (Lib.) Sacc. (2 strains), *F. compactum* (Wollenw.) Gordon, *F. culmorum* (W. G. Smith) Sacc. (3 strains), *F. detonianum* Sacc., *F. dimerum* Penz. (3 strains), *F. diversisporum* Sherb., *F. equiseti* (Corda) Sacc. (6 strains), *F. graminearum* Schw., *F. heterosporum* Nees ex Fr., *F. javanicum* Koorders, *F. lateritium* Nees ex Fr. (4 strains), *F. lini* Bolley (2 strains), *F. macroceras* Reinking, *F. merismoides* Corda, *F. orthoceras* App. and Wollenw., *F. orthoceras* var. *gladioli* McCulloch, *F. orthoceras* var. *longius* Wollenw., *F. orthoceras* var. *pisi* Linford, *F. oxysporum* Schlecht. ex Fr. (5 strains), *F. poae* (Pk.) Wollenw., *F. redolens* Wollenw., *F. roseum* Link, *F. sambucinum* Fuckel, *F. scirpi* Lamb. and Fautr. (2 strains), *F. scirpi* var. *acuminatum* (Ellis and Everh.) Wollenw., *F. semitectum* Berk. and Rav. (3 strains), *F. solani* (Mart.) Sacc. (4 strains), *F. sublunatum* Reinking, *F. trichothecioides* Wollenw., *F. tricinctum* (Corda) Sacc., *F. tumidum* Sherb. var. *caeruleum* Bugnicourt, *F. tumidum* var. *humi* Reinking, *F. vasinfectum* Atk.; *Gibberella cyanea* (Sollm.) Wollenw. (= *F. reticulatum* Mont.), *G. intricans* Wollenw. (= *F. equiseti*), *G. zeae* (Schw.) Petch (= *F. graminearum*) (4 strains).

These were grown on GAN medium and at intervals during 21 days growth samples were taken and culture filtrates sprayed on 'Meteor' pea seedlings. No culture filtrates induced increased shoot growth, though cultures of *G. fujikuroi* included as controls always induced increased growth. Slight scorching of foliage was occasionally noted from culture filtrates of *F. avenaceum* (2 strains), *F. culmorum* (1 strain), *F. dimerum* (1 strain) and *F. lateritium* (2 strains). Severe scorching of foliage, accompanied by marked retardation of stem growth and frequently by death of the plants, was consistently caused by culture filtrates from *F. equiseti* (2 strains), *F. scirpi* (1 strain) and *G. intricans* (1 strain). This result was all the more striking since, on morphological

grounds, Gordon (1952) considers *F. equiseti* (Corda) Sacc. and *F. scirpi* Lamb. and Fautr. to be synonymous, with *Gibberella intricans* Wollenw. as the perfect state. Further work was therefore concentrated on the more highly toxigenic cultures of *F. equiseti* (in the broad sense), namely *F. equiseti* (CMI. 35100), *F. scirpi* (CMI. 45490) and *G. intricans* (CBS, Wollenweber strain).

FURTHER STUDY OF TOXIN PRODUCTION BY *FUSARIUM EQUISETI*

Cultural conditions. For study of optimum cultural conditions for production of the phytotoxic substance or substances, and for the development of isolation techniques, a rapid bioassay was needed. Though culture filtrates showed slight antifungal and antibacterial activity this was not correlated with phytotoxicity, so that no conventional anti-microbial assay could be used.

TABLE I

Development of phytotoxic activity in cultures of F. scirpi (*CMI. 45490*)

Days growth at 25° C	GAN medium			Czapek–Dox			Czapek–Dox + yeast		
	PS units	Stem growth*	Foliar damage†	PS units	Stem growth	Foliar damage	PS units	Stem growth	Foliar damage
—	2	100	—	8	100	—	8	100	—
7	2,048	31	+++	8	105	—	128	50	+
11	8,192	29	+++	512	46	+++	512	39	+++
19	8,192	41	+++	512	74	++	512	70	++
25	8,192	30	+++	512	56	++	128	62	+
28	2,048	57	++	512	66	+	128	76	+

* As percentage of unsprayed control.
† Arbitrary assessment.

Assays based on growth inhibition and foliar damage to pea seedlings were very slow, requiring 3–4 days before any assessment of comparative toxicity could be made. Eventually an assay based on extension of pea internode sections (see Methods) was used, giving results in 24 hr. Culture filtrates were mixed with an equal volume of pH 6·1 phosphate buffer containing 20 μg./ml. indolylacetic acid (IAA); fourfold serial dilutions of this were then made, using as diluent phosphate buffer containing 10 μg./ml. IAA and 1 per cent. sucrose, so that all solutions assayed contained the same concentration of IAA. By determining the greatest dilution causing a significant reduction in extension of sections as compared with suitable controls, the activity can be expressed in PS (pea section) units analogous to the BA units of antifungal activity which we have used in other studies (Brian and Hemming, 1945); in this system if a dilution of 1 in 8 is the greatest dilution inhibiting section extension then the activity is expressed as 8 PS units and so on. The culture media themselves are mildly inhibitory, due to their high glucose content, but this does not confuse matters when assaying highly toxic culture filtrates (see Table I).

An example of the kind of result obtained is shown in Table I, where *F. scirpi* (CMI. 45490) was grown on three media (GAN, Czapek–Dox, and Czapek–Dox + 0·01 per cent. yeast extract) all initially containing 5 per cent.

glucose. Samples were taken at intervals and assayed both by the pea section assay and by spraying undiluted culture filtrates on 'Meteor' pea seedlings. It will be seen that the pea section assay gives the same general picture of development of activity as do observations on intact pea seedlings; indeed it is almost certainly more quantitative and is far more rapid. Of the three media, GAN is by far the best. On the basis of this and other experiments this type of medium was chosen for further work.

Isolation and characterization of toxic substances. Similarly, by use of the pea section assay it was found that the active materials could be extracted from culture filtrates into chloroform. Further purification procedures led to the conclusion that several phytotoxic substances were present, but that the most important of these was a colourless crystalline compound, diacetoxyscirpenol, m.p. 161–2°, $C_{19}H_{26}O_7$.

Diacetoxyscirpenol was obtained from *F. scirpi* (CMI. 45490), *F. equiseti* (CMI. 35100) and *G. intricans* (CBS); the best yields from these were of the order of 125 mg./l., 0·2 mg./l., and 50 mg./l. respectively. The isolation of the same substance from each of these cultures further confirms their taxonomic identity.

Two less-abundant phytotoxic substances with physical and chemical properties similar to those of diacetoxyscirpenol were also isolated. The first, m.p. 135–6°, $C_{19}H_{24}O_9$, was obtained from *F. scirpi* (CMI. 45490) and *G. intricans* (CBS). The second, m.p. 185–8°, probably $C_{21}H_{28}O_{10}$, although other possible molecular formulae cannot be excluded, was isolated from *F. scirpi* (CMI. 45490). They were obtained in yields of about 2 mg./l. and 1 mg./l. respectively.

In a typical experiment the culture filtrate (43 l.) from *F. scirpi* (CMI. 45490), grown on GAN medium for 12 days, was extracted at the natural pH (3·5) with chloroform (3×4 l.). A solution of the red oily extract (3·0 g.) in ether was filtered and set aside giving crystalline material (*A*) (1·15 g., m.p. 155–60°) and a residue (*B*). (*A*) was recrystallized from ether or ethyl acetate giving *diacetoxyscirpenol* in prisms, m.p. 161–2°, $[\alpha]_D^{19}+20°$ (*c*, 1·0 in acetone). (Found: C, 61·9, 62·1; H, 7·2, 7·2; CH_3CO, 23·85, 25·35; C—Me, 10·0, 10·9; Active H, 0·34 per cent.; *M* (Rast), 366. $C_{19}H_{26}O_7$ requires C, 62·3; H, 7·15; $2CH_3CO$, 23·5; 3C—Me, 12·3; 1 Active H, 0·27 per cent.; *M* 366·4.) Since diacetoxyscirpenol is optically active the unit cell is non-centrosymmetric. It was orthorhombic and had $a = 8{\cdot}25$, $b = 10{\cdot}60$, $c = 21{\cdot}24$ Å. The density, determined by flotation in cadmium borotungstate solution, was 1·316 g. cm.$^{-3}$. Hence *M*, assuming four molecules per unit cell, is 368±1 per cent. The ultra-violet spectrum (in ethanol) showed end-absorption only. The infra-red spectrum showed bands at ν_{max} (in Nujol) 3,415, 3,350 (OH); 1,739 (ester C=O); 1,681 (C=C); 1,241 (ester C—O); 820 cm.$^{-1}$ ($R_1R_2C{=}CHR_3$): (in $CHCl_3$) 1,740, 1,728, 1,250, 1,229 cm.$^{-1}$.

Diacetoxyscirpenol contained no alkoxyl groups. It was unsaturated to potassium permanganate in acetone and gave a pale blue colour with tetranitromethane in chloroform. Microhydrogenation with a palladium–carbon

catalyst resulted in the uptake of 1·04 mol. hydrogen, giving a product which was saturated to permanganate.

Diacetoxyscirpenol gave a violet colour with the Ehrlich reagent (*p*-dimethylaminobenzaldehyde and concentrated hydrochloric acid).

The *acetate* (triacetoxyscirpene) formed prisms, m.p. 124–5° $[\alpha]_D^{23}+44°$ (*c*, 1·0 in acetone) from ethanol. (Found: C, 61·6; H, 7·0; CH_3CO, 30·9. $C_{21}H_{28}O_8$ requires C, 61·6; H, 6·9; $3CH_3CO$, 31·6 per cent.) ν_{max} (Nujol) no OH absorption; 1,743, 1,250 (ester COO); 1,678, 830 cm.$^{-1}$ ($R_1R_2C{=}CHR_3$).

The *tosylate* crystallized from ethanol in needles m.p. 163°. (Found: C, 59·95; H, 6·2; S, 6·05; CH_3CO, 17·8. $C_{26}H_{32}O_9S$ requires C, 60·0; H, 6·2; S, 6·15; $2CH_3CO$, 16·55 per cent.)

The residue (*B*) in benzene (150 ml.) was chromatographed on a column of alumina (pH 4: grade II: 85 g.) in ultra-violet light. After a series of gummy fractions (-ve Ehrlich test) from non-fluorescent bands had been eluted with benzene (300 ml.) and benzene-methanol (200:1, 300 ml.), the latter eluant (100 ml.) brought down a blue fluorescent band. Fractional crystallization of the gummy product (0·97 g.) from ether gave diacetoxyscirpenol (0·43 g., m.p. 158–60°) and needles (from ether or ethyl acetate), m.p. 135–6° $[\alpha]_D^{25}+72°$ (*c*, 1·0 in acetone) of a *substance*. (Found: C, 57·7, 57·6; H, 6·1, 6·15; CH_3CO, 24·4, 24·4, 21·3; C—Me, 10·2. $C_{19}H_{24}O_9$ requires C, 57·6; H, 6·1; $2CH_3CO$, 21·7; 3C—Me, 11·4 per cent.) ν_{max} (Nujol), 3,450, 3,265 (OH); 1,740 (ester C=O); 1,689, 1,630 (C=C—C=O); 1,670 (C=C?); 1,235 (ester C—O). λ_{max} (in ethanol) 221 mμ ϵ, 7,250. Like diacetoxyscirpenol the compound contained no alkoxyl groups, was unsaturated to permanganate and gave a reddish-violet colour with the Ehrlich reagent.

Continued elution of the column with benzene-methanol (200:1, 100 ml.) gave a gum (0·34 g.) which crystallized from ether in needles (70 mg.) of a *substance* m.p. 185–8°. (Found: C, 57·6; H, 6·5; CH_3CO, 30·8. $C_{21}H_{28}O_{10}$ requires C, 57·3; H, 6·4; $3CH_3CO$, 29·3 per cent.) ν_{max} (Nujol) 3,425, 3,360 (shoulder) (OH); 1,741 (ester C=O). The ultra-violet spectrum showed end-absorption only.

Chloroform extraction of the culture filtrate (34 l.) from *G. intricans* (CBS) gave a gum (3·85 g.) from which diacetoxyscirpenol (1·6 g. m.p. 161–2°) and the substance m.p. 135–6° (50 mg.) were obtained by fractional crystallization from ether. Similar extraction of a culture filtrate (25 l.) of *F. equiseti* (CMI. 35100) furnished diacetoxyscirpenol (6 mg.).

We conclude that the principal toxin, $C_{19}H_{28}O_7$, is a diacetoxy alcohol containing one trisubstituted ethylenic double bond and (excluding acetyl residues) one C—Me group. Five of the seven oxygen atoms are accounted for in the hydroxyl and ester groups; the remaining two oxygen atoms are assumed to be present in ether linkages. For convenience, the $C_{15}H_{22}O_2$ skeleton obtained by removal of the acetoxyl and hydroxyl substituents has been given the trivial name *scirpene* from which the name *diacetoxyscirpenol* is derived. Consideration of the formula of diacetoxyscirpenol shows that it is a tetracyclic compound.

Hydrolysis of diacetoxyscirpenol. Hydrolysis of diacetoxyscirpenol with dilute alkali proceeded readily at room temperature with the formation of acetic acid (2 mols.) and an alcohol, scirpentriol. The same compound is obtained, among others, on hydrolysis with dilute mineral acids. Sodium hydroxide (10 ml., 0·1066 N) was added to a solution of diacetoxyxcirpenol (200 mg.) in ethanol (2·5 ml.) at room temperature. Aliquots were withdrawn at intervals and titrated against 0·1 N hydrochloric acid. 1 Mol. alkali was consumed after 1 min. and a second mol. during the next hour. Sodium hydroxide (10 ml.) was then added and the solution was kept for 24 hr. No further alkali was consumed. Continuous extraction of the neutral solution with chloroform for 96 hr. gave a yellow glass (138 mg.) which crystallized from ethyl acetate in prisms (102 mg.), m.p. 193°, $[\alpha]_D^{23}$ O±3° (*c*, 1·27 in acetone) of *scirpentriol.* (Found: C, 63·8; H 7·9; C—Me, 5·1, 5·2. $C_{15}H_{22}O_5$ requires C, 63·8; H, 7·85; 1 C—Me, 5·3 per cent.) ν_{max} (Nujol) 3,480, 3,455, 3,405 (OH); 1,676, 830 cm.$^{-1}$ ($R_1R_2C{=}CHR_3$).

Acetylation of scirpentriol with acetic anhydride in pyridine gave triacetoxyscirpene, identical (m.p. and mixed m.p. 124°) with the material obtained by acetylation of diacetoxyscirpenol.

BIOLOGICAL ACTIVITY OF THE ISOLATED TOXINS

In this study more attention has been devoted to diacetoxyscirpenol, the major metabolic product, than to the other two phytotoxic substances isolated from culture filtrates. In view of the ease with which the hydrolysis of diacetoxyscirpenol proceeds *in vitro*, yielding scirpentriol, it was considered to be a potentially possible reaction in living systems; accordingly, in the study of the biological activity of diacetoxyscirpenol, comparisons with scirpentriol were frequently made.

Toxicity to shoots of intact plants. Aqueous solutions of diacetoxyscirpenol were made at 0·1, 1, and 10 μg./ml., with 0·1 per cent. Tween 20 and 1 per cent. glycerol, and these were sprayed to run-off on seedlings of various species. A just-detectable inhibition of stem elongation was produced in peas (vars. 'Meteor' and 'Improved Pilot') by 1 μg./ml. and the foliage was noticeably scorched. At 10 μg./ml. stem extension was severely reduced, leaves were severely scorched, and many plants died (Fig. 1). Similar damage was done to lettuce ('May King' and 'All-the-year-round') and to winter tares. A characteristic effect on the latter plant was the killing of the apical bud and development of new shoots from axillary buds. Scorching and stunting of cress and tomato were caused by 10 μg./ml. diacetoxyscirpenol, but lower doses were without effect. Mustard, wheat (var. 'Victor'), beetroot, and carrots were unaffected by 10 μg./ml. Though highly toxic to pea plants, diacetoxyscirpenol does not appear to be translocated. Doses ranging from 0·001 μg. to 10 μg. were applied to the first true leaf of 'Meteor' pea seedlings. Though local lesions were produced, little or no effect on stem growth or on other leaves could be detected.

Scirpentriol has no effect on peas when applied as a spray at 10 μg./ml., but at 100 μg./ml. it reduced stem growth and caused scorching of foliage.

FIG. 1. 'Improved Pilot' pea: on right sprayed with 10 μg./ml. diacetoxyscirpenol, on left untreated control.

However, even at that dose it was less toxic than diacetoxyscirpenol at 10 μg./ml.

The substance m.p. 135–6° caused scorching of foliage and slight inhibition of stem growth at 10 μg./ml., and more severe effects at 100 μg./ml., but at the latter concentration the effect produced was less than that produced by 10 μg./ml. diacetoxyscirpenol. The substance m.p. 185–8° was slightly more

phytotoxic, a concentration of 100 μg./ml. producing an effect equal to that of 10 μg./ml. diacetoxyscirpenol.

Effect on growth of roots of cress seedlings. Diacetoxyscirpenol and scirpentriol do not differ so markedly in their toxic effects on cress root growth as they do in the other systems already discussed. Diacetoxyscirpenol is non-inhibitory at 2·5 μg./ml., reduces root extension to 50 per cent. of normal at 10 μg./ml., and prevents root growth at 100 μg./ml. Scirpentriol also almost completely inhibits root growth at 100 μg./ml., but is rather less inhibitory

TABLE II

Effect of diacetoxyscirpenol on cress root growth

[Mean length (mm.) after 4 days' growth at 25° C.]

Diacetoxyscirpenol (μg./ml.)	Expt. 1	Expt. 2	Mean
0 (control)	26·0	27·6	26·8
0·0006	26·7	27·1	26·9
0·0025	26·1	27·4	26·7
0·01	27·8	32·9†	30·3*
0·04	30·6†	32·5†	31·5†
0·16	33·2†	31·1*	32·2†
0·625	29·3*	33·1†	31·2†
2·5	29·6*	30·4	30·0*

Significantly different from control: * ($P = 0·05$), † ($P = 0·01$)

than diacetoxyscirpenol at 10 μg./ml. There is, nevertheless, a striking difference in the activity of the two substances: at sub-inhibitory levels diacetoxyscirpenol actually increases root extension (Table II), whereas scirpentriol does not. This stimulation has been consistently produced in many experiments.

Stimulation of root growth is sometimes interpreted as an anti-auxin effect. We have carried out numerous factorial experiments, with combinations of various levels of diacetoxyscirpenol and indolylacetic acid; in most cases, as in the experiment summarized in Table III, there was no evidence of interaction, the effects of the two substances being additive. In occasional experiments there was a significant interaction, diacetoxyscirpenol significantly reducing the toxic effect of 0·5 μg./ml. indolylacetic acid. Since this result could not be repeated at all consistently, it seems safest to treat this indication of anti-auxin activity with reserve. Gibberellic acid at concentrations of 1–10 μg./ml., which by itself has no effect on cress root growth (Brian, Hemming, and Radley, 1955), does not affect the response to diacetoxyscirpenol. Similarly, we have detected no interaction between diacetoxyscirpenol and cysteine. Though in our experiments we have obtained no consistent direct evidence of anti-auxin activity, the suspicion of such an effect suggested by the stimulation of root growth has led us to examine the possibility of such an effect, or of an anti-gibberellin effect, in other systems.

The substance m.p. 135–6° inhibited cress root growth more severely than

diacetoxyscirpenol at 10 μg./ml. At lower concentrations (0·2–0·6 μg./ml.) it stimulated root growth slightly, the effect being smaller than that of diacetoxyscirpenol. The substance m.p. 185–8° was even more inhibitory to cress roots, causing a marked inhibition at 0·6 μg./ml.; it did not stimulate root growth at sub-toxic concentrations.

TABLE III

Effect of diacetoxyscirpenol and indolylacetic acid (IAA) on cress root growth

[Mean length (mm.) after 4 days' growth at 25° C.]

IAA (μg./ml.)	Diacetoxyscirpenol (μg./ml.)				Mean
	0	0·02	0·1	0·5	
0	27·0	28·5	32·3	36·1	31·0
0·02	22·8	23·3	25·6	29·4	25·3
0·1	16·7	19·7	21·5	21·9	20·0
0·5	15·8	16·6	19·7	21·3	18·4
Mean	20·6	22·0	24·8	27·2	

Significant difference (marginal means): 2·2 ($P = 0·05$), 2·9 ($P = 0·01$)
IAA × diacetoxyscirpenol interaction not significant ($P > 0·05$)

TABLE IV

Effect of combinations of diacetoxyscirpenol and indolylacetic acid (IAA) on extension of pea internode sections

[Mean increment (mm.) of 5-mm. sections in 24 hr. at 15° C.]

IAA (μg./ml.)	Diacetoxyscirpenol (μg./ml.)				
	0	0·008	0·04	0·2	1·0
0	1·8	1·8	1·4	1·2	0·8
5	3·2	3·0	2·8	1·8	1·1
10	4·1	3·6	2·9	2·0	1·2
20	3·6	3·8	3·1	2·2	1·2

Significant difference: 0·31 ($P = 0·05$); 0·40 ($P = 0·01$)

Pea internode section extension. Extension is significantly reduced by 0·04 μg./ml. diacetoxyscirpenol (Table IV). At this concentration no discoloration of sections was seen, but concentrations of 1 μg./ml. and higher caused considerable browning of the cut ends of the sections. In a factorial experiment in which diacetoxyscirpenol and IAA are combined at various concentrations there is evidence of an IAA : diacetoxyscirpenol interaction ($P < 0·001$). Inspection of the data in Table IV shows that this interaction is due to the effects of the two substances being less than additive; in fact, diacetoxyscirpenol blocks the response to IAA. We have been able to obtain no evidence of any competitive relation between the two substances and it does not seem appropriate to characterize the activity of diacetoxyscirpenol as auxin-antagonism. Similarly, it blocks the response of sections to gibberellic acid. Its toxicity is not reduced by cysteine.

Scirpentriol is a much less active inhibitor, 10 μg./ml. being necessary to cause a significant reduction in extension. Because of its structural similarity

to diacetoxyscirpenol, scirpentriol might act as an antagonist of diacetoxyscirpenol; in numerous factorial experiments the effects of the two substances have always been found to be strictly additive.

The substance m.p. 135–6° was less inhibitory than diacetoxyscirpenol; at 1 μg./ml. it produced an effect equal to that of 0·1 μg./ml. diacetoxyscirpenol. The substance m.p. 185–8° was a more active inhibitor, but noticeably less so than diacetoxyscirpenol.

TABLE V

Effect of combinations of diacetoxyscirpenol and gibberellic acid (GA) on expansion of bean leaf-disks

[Final diameter (mm.) of disks, of initial diameter 5 mm., after 48 hr. at 15° C. in high-intensity light]

GA (μg./ml.)	Diacetoxyscirpenol (μg./ml.)			
	0	0·25	0·5	1·0
0	7·8	7·1	7·0	6·8
0·1	8·6	7·6	7·1	6·9
1·0	9·0	7·7	7·1	7·0
10·0	8·9	7·6	7·2	6·9

Significant difference: 0·33 ($P = 0·05$); 0·45 ($P = 0·01$)

Bean leaf-disk expansion. Bean leaf-disks under the conditions described are unaffected by auxins, but respond to gibberellins by increased expansion. Diacetoxyscirpenol prevents expansion and blocks the response to gibberellic acid (Table V); it causes serious discoloration of the mesophyll tissue at concentrations of 0·1 μg./ml. and above. Cysteine also stimulates expansion of such disks, but less markedly than gibberellic acid; it does not reduce the toxicity of diacetoxyscirpenol.

Scirpentriol is much less toxic to leaf tissue than diacetoxyscirpenol, disk expansion being reduced slightly but significantly at 5 μg./ml. but not at lower concentrations; it does not cause discoloration of leaf tissues even at 10 μg./ml. The effects of scirpentriol and of diacetoxyscirpenol are additive; there is no interaction.

Antifungal and antibacterial activity. Spore germination tests were carried out at pH 3·5 and pH 7·0, using spores of *Absidia glauca*, *Aspergillus niger*, *Botrytis allii*, *Fusarium* spp., *Mucor erectus*, *Myrothecium verrucaria*, several *Penicillium* spp., *Stemphylium radicis*, *Thamnidium elegans*, and *Trichoderma viride*. Germination of *Penicillium digitatum* was inhibited by 100 μg./ml. diacetoxyscirpenol, but all others were unaffected, as measured by percentage germination. With all fungi, germ-tube extension was retarded by diacetoxyscirpenol in concentrations of 6·25–50 μg./ml.

No inhibitory effect was noted at 200 μg./ml. with *Bacillus subtilis*, *Salmonella Dublin*, and *Escherichia coli*.

Thus diacetoxyscirpenol has little antibacterial or antifungal activity.

Toxicity to animals. Diacetoxyscirpenol is highly toxic to animals. We are

indebted to Dr. A. A. B. Swan of the Industrial Hygiene Research Laboratories, Imperial Chemical Industries Ltd., for the following information. The single dose intraperitoneal LD_{50} in female albino rats is 0·75 mg./kg. (confidence interval for $P < 0{\cdot}05$: 0·67 to 0·81 mg./kg.) and the oral LD_{50} is 7·3 mg./kg. (confidence interval for $P < 0{\cdot}05$: 5·9 to 8·9 mg./kg.). Thus its systemic toxicity is high. It has, in addition, a powerful local action causing necrosis of the skin and inflammation of the conjunctiva and corneal damage in the eye. There is an interval of at least 5–6 hr. between injection or application of diacetoxyscirpenol and the production of an observable effect. Since this interval is not reduced even when a massive dose (25 mg./kg.) is given, it suggests that a metabolite of diacetoxyscirpenol may be responsible for the toxicity.

Scirpentriol, though much less phytotoxic than diacetoxyscirpenol, is of comparable toxicity to female rats; the single dose intraperitoneal LD_{50} being 0·81 mg./kg. (confidence interval for $P < 0{\cdot}05$: 0·70–0·94 mg./kg.). The intraperitoneal LD_{50} of substance m.p. 135–6° and of substance m.p. 185–8° was, in both cases, 1·21 mg./kg. (confidence interval for $P < 0{\cdot}05$: 0·93–1·58 mg./kg.). The delay between application and appearance of toxic symptoms noted for diacetoxyscirpenol was not seen with these other three substances; the animals looked in poor condition within 30 min. of injection.

DISCUSSION

The general impression given by the data presented above is that diacetoxyscirpenol is a generally toxic substance, highly toxic to plants and animals. It is noteworthy, nevertheless, that it is of low antimicrobial activity.

The marked stimulation of root growth at sub-toxic concentrations is of particular interest. Very low concentrations of auxin may stimulate root extension of intact plants of some species, but the effect is usually difficult to demonstrate (Åberg, 1957; Torrey, 1956). More striking stimulations are produced by some 'anti-auxins' at higher concentrations (Torrey, 1956). Diacetoxyscirpenol stimulates root growth in the same concentration range as the anti-auxins, but we have been able to obtain no evidence that its effects are due to true auxin-antagonism. Root growth stimulation by salts of some simple organic acids, such as acetate, have been reported (Cooil, 1951). As we have mentioned earlier, it seemed possible that diacetoxyscirpenol might be hydrolysed in plant tissues to give scirpentriol and acetate, and in view of the reported stimulatory effect of acetate, this production of acetate might account for the stimulatory effect of diacetoxyscirpenol. This is not the case; under our experimental conditions neither acetate nor scirpentriol enhance root growth.

There has been one other record of production of root growth-promoting substances by *Fusarium* spp. (Venkata Ram, 1959) but in that case the effect was attributed to certain amino-acids.

Fusarium equiseti is a plant parasite, and the fact that it is capable of producing highly phytotoxic metabolic compounds *in vitro* carries with it the

possibility that *in vivo* production of the same compounds may be responsible for some of the symptoms of parasitic attack by *F. equiseti*. It seems to us quite probable that *F. equiseti* will produce diacetoxyscirpenol, and the other phytotoxic substances described in this paper, in any plant tissues which it may invade, since conditions for their production do not appear to be at all critical. However, *F. equiseti* is usually a weak parasite, and there is little suggestion that any of the characteristic symptoms of the diseases which it causes are toxigenically induced.

The marked toxicity to animals of diacetoxyscirpenol, and of the substances m.p. 135–6° and 185–8°, does suggest that they may be involved in the toxicoses caused by feeding grain infected with *Fusarium* spp. (Ainsworth and Austwick, 1959; Prentice, Dickson, and Dickson, 1959).

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Changes in the Pectin (Uronic Acid) Content of Storage Tissue Disks

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SUMMARY

Changes in the pectin (uronic acid) content are reported for disks of carrot, swede turnip, sugar-beet, red beet, and potato maintained in aerated, running tap water at three temperatures for periods of up to 20 days. Under certain conditions, synthesis of uronic acids, as estimated by a micro-decarboxylation method, occurs and there are changes in the relative amounts of water-soluble and water-insoluble constituents of the tissues. The increases in water-insoluble material and the periods when the maximum changes occur are discussed in relation to the corresponding changes in dry weights of the tissues.

INTRODUCTION

THE present investigation is part of a larger study of the metabolic changes induced in disks of carrot, swede turnip, sugar-beet, red beet, and potato maintained in aerated, running tap water. The apparatus and experimental technique have been described (MacDonald and Knight, 1958) and changes in respiration and sugar content (MacDonald and DeKock, 1958) and mineral composition have been investigated (MacDonald, DeKock and Knight, 1960). The sensitivity of the disks to respiration inhibitors has been reported by MacDonald (1959).

Some evidence has already been obtained which suggests that slicing initiates a renewal of growth in the dormant storage tissue. The disks increase in size and it seemed probable that, along with changes in the other carbohydrates, there would be changes in the content and nature of the pectic substances, especially if new cells were being formed incorporating calcium pectate in their middle lamella.

Although disks of storage tissue have been used extensively in plant physiology, particularly in studies of salt uptake, very little attention has been given to their content of uronic acids or pectic substances. This is surprising, since the free carboxylic acid groups of these constituents are capable of exchanging cations with the surrounding medium. This aspect was an additional reason for undertaking the present study, owing to the authors' interest in the cation-exchange capacity (C.E.C.) of plant roots (Crooke, 1958) and its possible relationship to ion absorption. The changes in C.E.C. of similar disks of the same five tissues with time and their relationship to the content of uronic acids have been reported (Crooke, Knight, and MacDonald, 1960).

There is an extensive literature on the pectic substances in fruits and storage organs as related to quality and the alterations in these substances during ripening and maturation. In general, insoluble substances decrease and soluble components increase during this period, although this is not always the case (Ahmed and Scott, 1958; Bettelheim and Sterling, 1955; Dastur and Agnihorti, 1934). Similar changes are induced by cooking or steaming of root vegetables (Simpson and Halliday, 1941).

MATERIALS AND METHODS

Material. Disks from the storage organs of carrot (*Daucus carota*), swede (*Brassica napus*), sugar-beet and red beet (*Beta vulgaris*), and potato (*Solanum tuberosum*) were prepared as described by MacDonald and Knight (1958) and transferred to the disk-washing unit, in which they were maintained in constantly aerated, running tap water. There was never any evidence of deterioration due to bacteria. The temperatures used were 25° C., 15° C., and less than 2° C. (obtained by means of a refrigerating coil immersed in the

TABLE I

Design of experiments

		Carrot	Turnip	Sugar-beet	Red beet	Potato
Temperature of storage . .	° C.	2, 15, 25	2, 15, 25	2, 15, 25	2, 15, 25	2, 15, 25
Disk thickness . . .	mm.	1, 1, 1	1, 1, 1	0·5, 0·5, 0·5	0·5, 0·5, 1	1, 1, 1
Length of experiment .	Days	21, 18, 10	16, 16, 10	14, 11, 10	10, 11, 9	7, 7, 7

water jacket surrounding the disk-washer). The first sample of disks was generally taken 2 hours after cutting and subsequent samples were taken daily or at suitable intervals. The wet weight of 10 freshly cut disks 1·1 cm. in diameter and 0·1 cm. thick was always about 1·0 g. for the five tissues and therefore 10 disks may be conveniently regarded as weighing 1 g. Where a thickness of 0·5 mm. has been used the results are reported on a 20-disk basis i.e. all measurements are calculated as per gram of initial fresh weight. Table I sets out the various experiments giving details of duration and temperature used.

Length of experiment. This was governed to a large extent by the temperature of storage and, to a lesser degree, the disk thickness. One-mm. disks of potato, for example, disintegrated rapidly at 25° C. and experiments with this tissue were restricted to 7 days.

Dry weights. Disks were dried in an oven overnight at 80° C. The disks were allowed to drain on filter-paper without blotting before transfer to tubes for drying in the oven. Steward, Stout and Preston (1940) found mechanical losses of pectic substances by blotting their potato disks between paper.

Uronic acid content. The estimation was made by the decarboxylation method of Lefèvre and Tollens, as modified by Tracey (1948) for milligram quantities. Less than 50 mg. of dried material (approximately five disks) was heated in a small sealed ampoule with 0·5 ml. of 12 per cent. HCl (w/w) at

111° C. for 5 hours. The CO_2 evolved was measured in a Van Slyke apparatus. A standard glucurone (25 per cent. yield of CO_2) was included in each batch of 12 samples.

Extraction of total pectic substances. Dried milled material was extracted with hot water in the presence of a calcium complexing agent ('Calgon'). The filtered extract was poured into acidified ethanol (Owens *et al.*, 1952). The resultant alcohol precipitate was examined for its content of uronic acids by decarboxylation and for identification of individual uronic acids and sugars by paper chromatography after acid hydrolysis (N. H_2SO_4 for 5 hours).

Paper chromatography. Unidimensional paper chromatography was carried out by the descending technique using Whatman No. 1 paper with butan-1-ol:acetic acid:water 4:1:5 (v/v/v) as a solvent. After development and drying in a current of hot air the papers were sprayed with naphthoresorcinol-hydrochloric acid or with *p*-aminobenzoic acid (v. Euler and Hasselquist, 1953) to reveal glucuronic and galacturonic acids or glucuronic acid respectively, or with aniline sulphate to reveal sugars.

Separation of cell-wall materials. A method was sought by which the cell-wall material could be rapidly and easily separated from the cell contents. The changes in uronic acid content of cell walls might give a more significant indication of the formation of additional cell-wall materials than changes in the uronic acid content of the whole tissue. It was found that a short time of boiling (5 minutes) with 20 disks to 40 ml. water was sufficient to kill the tissue and release the cell contents but not long enough to disintegrate the disks. The dry weight and uronic acid content of boiled disks were determined as above for fresh or unboiled disks.

Estimation of cation-exchange capacity. The C.E.C. of disks was determined by titration to pH 7 with KOH (Crooke *et al.*, 1960).

Expression of results. The method of expression of the results of uronic acid (or pectic) estimations is commented on strongly by Kertesz (1951, pp. 282–3). The percentage content of any one component may change merely because the proportion of accompanying materials changes, without actual gain or loss of the component itself. The simplest method of overcoming these effects is to express the analytical result on the single-unit basis. This recommendation has been followed here and results are expressed per unit weight of fresh tissue on day 0 of each experiment.

RESULTS

Table II summarizes the relationship between the dry weights of the whole disks and the residues after loss of the cell contents on boiling. Losses on boiling of 60–70 per cent. for four of the tissues, with potato losing only 20 per cent., are typical values for these materials. The daily losses in dry weight of the unboiled disks with time of storage can be expressed on a percentage basis since the decreases are approximately linear over the period of the experiment.

The changes in content of uronic acids and in dry weight of the 'hot water

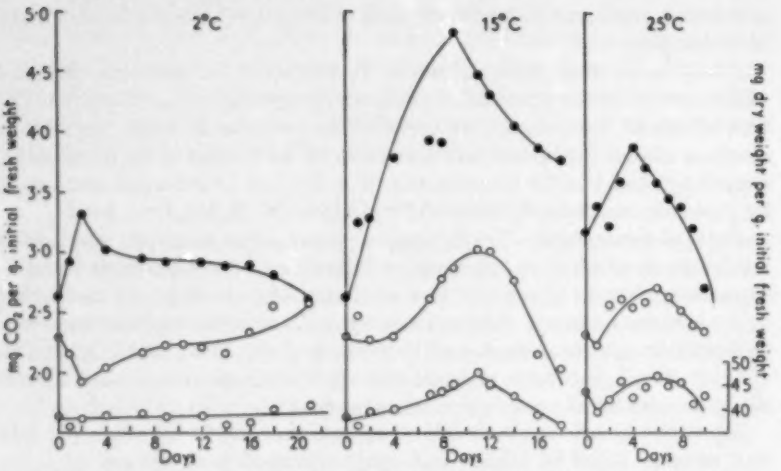

FIG. 1. Carrot 2°, 15°, and 25° C.: yield of CO_2 on decarboxylation from 1 g. initial fresh weight for fresh disks (●) and boiled disks (○). Lowest curve shows dry weight of boiled disks from 1 g. initial fresh weight (○).

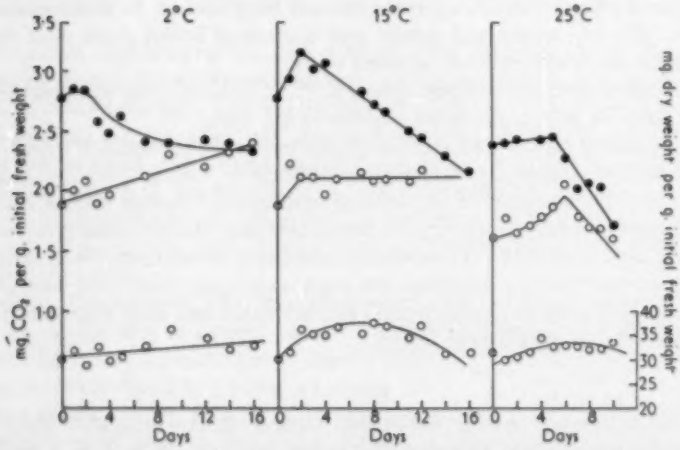

FIG. 2. Turnip at 2°, 15°, and 25° C.: yield of CO_2 on decarboxylation from 1 g. initial fresh weight for fresh disks (●) and boiled disks (○). Lowest curve shows dry weight of boiled disks from 1 g. initial fresh weight (○).

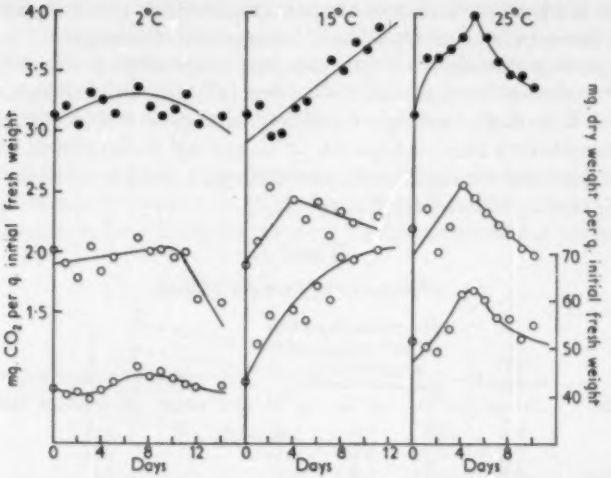

FIG. 3. Sugar-beet at 2°, 15°, and 25° C.: yield of CO_2 on decarboxylation from 1 g. initial fresh weight for fresh disks (●) and boiled disks (○). Lowest curve shows dry weight of boiled disks from 1 g. initial fresh weight (○).

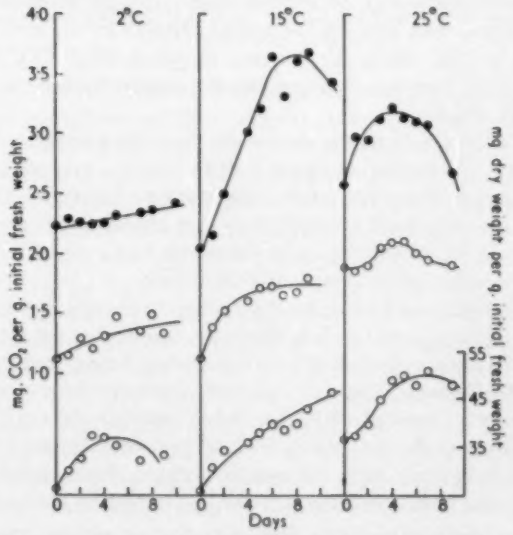

FIG. 4. Red beet at 2°, 15°, and 25° C.: yield of CO_2 on decarboxylation from 1 g. initial fresh weight for fresh disks (●) and boiled disks (○). Lowest curve shows dry weight of boiled disks from 1 g. initial fresh weight (○).

insoluble fraction' of the tissues are presented in Figs. 1–5, each figure being divided into 3 parts corresponding to temperature of storage.

Changes in pectic substances with time and temperature. (*a*) *Carrot.* Inspection of the curves shows that at 2° C., when little metabolic change would be expected, there is no evidence of a definite change in boiled dry weight but pectin curves meet after 21 days. At 15° C. and 25° C. the curves for 'boiled' pectin values and the dry weight pass through a maximum about the same day, day 11 at 15° C. and day 6 at 25° C.

TABLE II

Changes in dry weight of disks

Tissue	Dry weight* in mg.	Daily percentage loss in dry weight at temperature: 2° C.	15° C.	25° C.	Dry weight of boiled disks* / Dry weight of unboiled disks* × 100
Carrot .	101	0·8	1·6	3·6	37
Turnip .	93	2·3	2·6	4·1	33
Sugar-beet	136	nil	1·8	4·0	37
Red beet	106	0·8	1·6	3·3	31
Potato .	154	1·7	6·0	7·5	82

* Average values corresponding to 1 g. initial fresh weight on day 0.

Although the maxima in these curves for pectin in boiled disks and dry weight occur at the same time during the experiment, the increase in weight is not accounted for, except to a minor degree, by the increase in weight of pectic substances. The increase for boiled disks at 15° C. over days 0–11 is 9·3 mg. dry weight, while the increase in pectin (mg. $CO_2 \times 4{\cdot}4$) is only 3·1 mg. At 25° C. between days 0–6 the dry weight increase is about 6 mg. and the pectin 1·8 mg.

(*b*) *Turnip.* Fig. 2, A–C, shows that at the three temperatures the curves for pectin content of unboiled and boiled disks tend to converge, the point of convergence being dependent on the temperature of storage. Compared with carrot, there are only small increases in pectin content and dry weight and it is difficult to relate the maximum in the pectin curve at 25° C. to a definite maximum in the dry weight graph of boiled disks.

(*c*) *Sugar-beet and red beet.* A general pattern of changes is found similar to those in carrot (Figs. 3, and 4 A–C). However, convergence of curves of pectin content of boiled and unboiled disks is not evident owing to the shorter duration of the experiments. There is a general correspondence of change in dry weight with pectin content of boiled disks, especially at 25° C., where the maxima occur about day 4 to day 6 for both varieties of beet. For sugar-beet the change in weight of pectic substances between day 0 and day 5 is about 2·0 mg. while the corresponding increase in dry weight of the same disks is 12·5 mg.

(*d*) *Potato.* In Fig. 5 only the results of the 25° C. experiment are shown; changes at 2° C. and 15° C. were similar but of smaller magnitude. There is

no evidence of an increase in pectin nor of a decrease or change in the small amount of water-soluble substances. The weights of both boiled and unboiled disks can be given on the same scale for this tissue, since boiling reduced the dry weight by only 20 per cent. After 7 days the unboiled potato disks have lost 52 per cent. of their initial weight and the boiled disks have lost 59 per cent.

Decarboxylation method. A study was made of the influence of interfering substances normally present in plant material and capable of evolution of carbon dioxide under the conditions used for the estimation of uronic acids.

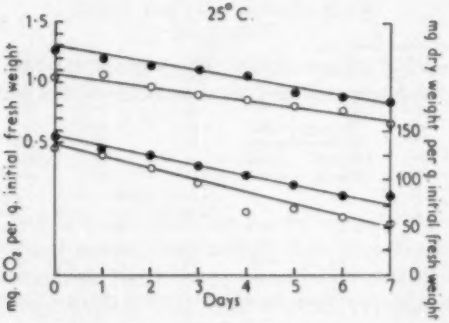

FIG. 5. Potato at 25° C.: yield of CO_2 from 1 g. initial fresh weight for fresh disks (●) and boiled disks (○). Lower lines show dry weight of fresh disks (●) and boiled disks (○) from 1 g. initial fresh weight.

Less than 1 per cent. CO_2 was evolved from pure samples of sucrose, fructose, glucose, galactose, xylose, cellulose, starch, oxalic acid, citric acid, and malic acid compared with 23 per cent. CO_2 from galacturonic acid. These values are similar to those quoted by Tracey (1948) and other workers. The polyphenol, chlorogenic acid, which has been the subject of recent studies with storage tissue (Lieberman, Craft, and Wilcox, 1959; Zucker and Levy, 1959), was examined and found to yield 5 per cent. CO_2 on decarboxylation. Ascorbic acid yielded 20 per cent. CO_2, which indicated that serious misinterpretation could be made of the origin of the carbon dioxide evolved if the tissues contained appreciable amounts of this substance. Spector (1956) quotes values for ascorbic acid in mg./100 g. fresh tissue for turnip (28), red beet (10), potato (17), and parsnip (18). On the same basis Olliver (1936) gives an average value for carrot of less than 4, and a rather higher figure for new potatoes of 34·8. Actual values for ascorbic acid by indophenol titration for our materials were in mg./100 g. fresh: sugar-beet (1), carrot (3), old potato (5), new potato (18), turnip (14). Even if storage tissue is assumed to have an average content as high as 20 mg. ascorbic acid/100 g. fresh tissue, then with 20 per cent. evolution of carbon dioxide there will be 0·04 mg. yield CO_2/g. fresh weight. The lower yields of CO_2 from the tissues in the

present investigation are generally about 1–2 mg./g. fresh weight, so the interference from ascorbic acid would be negligible (4 per cent.).

There is also the possibility of erroneous interpretation of the origin of the CO_2 evolved by the presence of a compound which gives a low yield of CO_2 (<1 per cent.) but is present in large amounts, e.g. sucrose in unboiled disks. The maximum sucrose content found in sugar-beet on day 0 after slicing is 12 per cent. of the fresh weight. Hence, with 0·5 per cent. evolution of CO_2, there is produced 0·6 mg. CO_2 per g. initial fresh weight or about 20 per cent. of the total evolved from all sources. All of the water-soluble interfering substances would be removed by the boiling procedure.

TABLE III

Comparison of yields of carbon dioxide from original material and from alcohol precipitates of disk extracts; and the composition of the acid hydrolysates

	CO_2 (per cent.)							
	Original material	Pectin extract	Disaccharides	Galactose	Glucose	Arabinose	Galacturonic	Glucuronic acid
Carrot	2·60	8·77	+	2+	0	3+	3+	0
Turnip	2·75	8·87	2+	2+	0	3+	3+	0
Sugar-beet	3·05	8·81	+	2+	0	4+	3+	0
Potato	1·30	1·24	2+	3+	3+	trace	trace	0

Intensity of spot on chromatogram on scale 0 (absent) to 4+ (very strong).

Extraction of total pectic substances. Since the decarboxylation method is not specific for pectic substances it was considered necessary to demonstrate their presence in substantial amounts in the experimental materials. The extraction method used is known to yield results between 90 and 100 per cent. of these obtained by direct anhydrouronic acid analysis of grapefruit, cranberries, raspberries, and carrots (Owens *et al.*, 1952).

Chromatography demonstrated large amounts of galacturonic acid and the absence of glucuronic acid (Table III). From the CO_2 figures it will be seen that in the extract a threefold concentration of the CO_2-yielding substances has been achieved, i.e. the alcohol precipitate contained up to 40 per cent. polygalacturonic acid. The other components of hydrolysis were all in keeping with the view that the alcohol precipitate from the water extract of the four storage tissues was largely pectic in nature. These results show that CO_2 yielded by boiled disks is very probably derived from pectic substances present in the disks.

In the case of potato, the low yield of CO_2 suggests that the alcohol precipitate is mainly starch and this is confirmed by the chromatographic results. Potato starch evolved 0·4 per cent. CO_2 during decarboxylation tests.

Effect of saponification. Pectic substances may occur in plant material in various degrees of esterification by methyl radicals. The degree of esterification as well as size of polymer is of importance in regard to extractibility of the substances by hot water. On day 4 of the 25° C. experiments additional samples of disks were analysed for pectic substances by the decarboxylation

method after treatment as follows: (*a*) unboiled, (*b*) boiled, (*c*) unboiled, saponified with sodium hydroxide, (*d*) boiled, then saponified as in (*c*). The method of saponification was that of Keller and Deuel (1957) in that 10 disks were treated with 100 ml. 0·1 N.NaOH and 1 N.NaCl for 2 hours at room temperature with occasional stirring. Table IV shows that there is a progressive reduction in dry weight from unboiled disks to boiled, unboiled/saponified, and boiled/saponified. The amounts of CO_2 evolved decrease markedly

TABLE IV

Dry weight and uronic acid content of disks before and after saponification

Treatment	Unboiled		Boiled		Unboiled/Saponified		Boiled/Saponified	
Dry weights and CO_2 per g. initial fresh weight	Weight in mg.	CO_2 in mg.	Weight in mg.	CO_2 in mg.	Weight in mg.	CO_2 in mg.	Weight in mg.	CO_2 in mg.
Carrot . . .	86	3·88	42	2·54	37	2·82	26	2·46
Turnip . . .	70	2·44	34	1·80	38	2·06	21	1·94
Sugar-beet . .	117	3·80	62	2·56	63	2·66	53	2·38
Red beet . . .	98	3·23	49	2·11	35	2·06	23	1·60
Potato . . .	104	1·05	67	0·86	32	0·86	21	0·89

from unboiled disks to boiled disks. It can be seen by the similarity of the results for boiled and unboiled/saponified disks that boiling in water and treatment in the cold with NaOH have the same general effect in removing a category of substance yielding CO_2. Both methods probably kill the cells and release the cell contents without significant attack on the cell-wall materials. Treatment by boiling followed by saponification, in which it might be expected that the NaOH solution would have greater access to attack cell walls, did not produce any significant change in content of pectic substances but only in dry weight.

Changes in saponifiable constituents with time in boiled disks. Cation exchange capacity measurements were made on boiled disks and boiled/saponified disks during the whole course of the experiments at 25° C. In the case of boiled/saponified disks, the acidic groups produced on saponification would be included in the measurement as well as the free acidic groups. Fig. 6, A–D, shows that these additional groups cause an increase of about 20 per cent. of the unsaponified values except in the case of potato, where the increase on saponification is negligible. The general trend with carrot, turnip, and the beets is for the component affected by saponification to disappear with time as shown by the convergence of the two curves.

Our C.E.C. method does not tell anything of the possible changes taking place in esterified compounds in the water-soluble fraction. In addition, it cannot be assumed that the acidic groups measured during the determination of cation exchange capacity are wholly accounted for by pectic substances. A fuller discussion of this aspect has been reported (Crooke *et al.*, 1960). It is sufficient to note that the percentage of esterified groups is small in the fraction of material left after boiling the disks and that there is a tendency for these esterified groups to disappear with time.

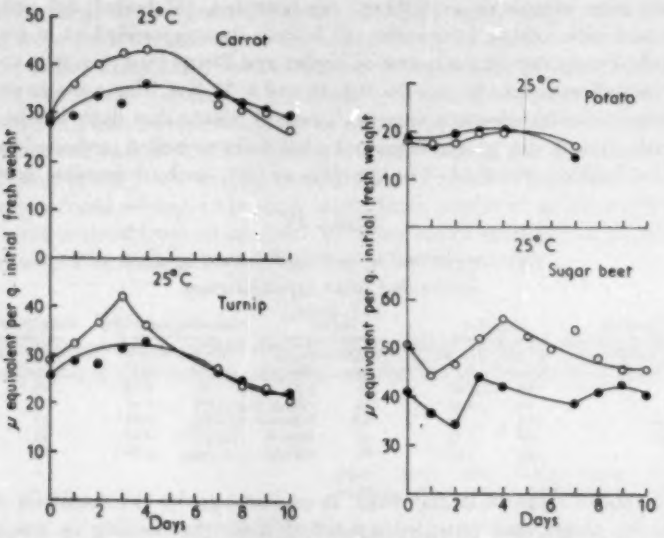

FIG. 6. Carrot, turnip, sugar-beet and potato at 25° C.: cation exchange capacity of saponified (○) and unsaponified (●) boiled disks.

TABLE V

Extraction of pectic substances from red beet disks

Treatment	Dry weight in mg. per g. initial fresh wt.	mg. CO_2 per g. initial fresh wt.	Pectin as percentage of dry weight
Fresh	83	3·56	18
Boiled in alcohol for 30 min.	63	3·04	21
Boiled in water for 5 min.	53	2·55	21
Boiled in alcohol then in water for 5 min.	51	2·41	21
" " " " " 30 "	48	1·96	18
" " " " " 1 hour	49	1·95	18
" " " " " 6 hours	48	1·33	12
" " " " " 12 "	36	0·84	10
Decalcified	51	2·63	22
" then boiled in water for 5 min.	50	2·53	22
" then extracted with 0·05 N. HCl	49	2·57	23
" " " " 0·05 N. NaOH	25	0·48	8

Successive extraction of pectic substances. Fractionation procedures for plant cells, in particular the cell-wall materials, have been reported by various workers (Jermyn and Isherwood, 1956: Ordin *et al.*, 1955). Non-polymeric substances are usually removed by boiling in aqueous alcohol followed by extraction of the cell-wall material with boiling water for periods of up to 12 hours to remove pectins. Water-insoluble pectic substances such as protopectin, calcium pectate, and polyuronide hemicelluloses are removed by

various acid and alkali treatments and the use of calcium complexing reagents such as ammonium oxalate. Table V shows various treatments which were performed on red beet disks from a secondary experiment which had been running for 7 days at 25° C. The decalcification of disks was accomplished by percolating 150 disks in a column overnight with 500 ml. of ammonium chloride—sodium acetate solution buffered to pH 3·5 (0·67 M. NH_4Cl: 0·33 M. sodium acetate) (Holden, 1950).

This treatment was effective as shown by chemical analyses; calcium was reduced from 247 to 73 μg. Ca per g. initial fresh weight. On the same basis K was reduced from 330 to 60 μg.; Na from 3980 to 65 μg. and Mg from 185 to 4 μg. The object of decalcification was to convert any calcium pectate to a more soluble form, but at the same time it is obvious that the cells were killed and plasmolysed by the salt solution.

Boiling in water for 12 hours or a short-term extraction in NaOH reduced the concentration of pectic substances in the residue from about 20 per cent. to 8–10 per cent.

It might be argued that certain of the extraction procedures did not proceed to completion since intact disks were used. In cases where disintegration occurred quantitative recovery was made by filtration. A test was made of red-beet tissue after maceration in a Waring blendor; results in mg. CO_2 per g. initial fresh weight were 3·96, 2·24, and 0·96 for (*a*) fresh tissue, (*b*) boiled in water for 5 minutes, and (*c*) boiled for 12 hours respectively. The relative contents confirm the values in Table V and show that subdivision of the material was not an important factor.

DISCUSSION

Levels of pectic substances. Before attempting to assess the meaning of the changes found in the uronic acid contents, a comparison should be made of the general levels found with those reported in the literature. The CO_2 evolved and measured by the micro-decarboxylation method cannot be related with certainty to any particular chemical constituent in the tissue without independent separation of compounds from the tissue and identification by specific tests. Even non-uronic acids evolve CO_2 (Tracey, 1948) and the possibility of these artefacts must be taken into account for each tissue individually. However, considering that the experimental material is storage tissue it will be assumed (for the purposes of this exploratory paper) that the CO_2 is evolved for the most part by decarboxylation of the combined galacturonic acid of pectic substances (Kertesz, 1951, p. 230). Keller and Deuel (1957) investigated the relationship of C.E.C. to content of pectic substances in roots of various crop plants. They state that they did not find any uronic acid other than galacturonic acid in hydrolysates of their plant roots.

Polyuronide hemicelluloses are found in greater proportions than pectic substances in materials such as straw and woody tissues (Bonner, 1950) and it would therefore seem unlikely that they would contribute significantly to the carbon dioxide analyses recorded here.

The corresponding weight of polygalacturonic acid is derived by multiplication of the weight of CO_2 by a factor of 4·4 (Kertesz, 1951, p. 36). The range of contents then becomes 0–20 mg. pectin/g. initial fresh weight. As a very approximate indication of the magnitude of the pectin content initially, it has been calculated for carrot, turnip, sugar-beet, and red beet that pectin is 10 per cent. of the dry weight of the unboiled disks and 20 per cent. of the weights of boiled disks. Our results fall within the ranges compiled from the literature by Kertesz (1951) and since these figures were obtained mostly by the gravimetric calcium pectate method, this concordance lends support to our view that predominantly pectic substances were being estimated by the decarboxylation method used here. It is less easy to extract figures from the literature for water-soluble pectic substances for comparison with our results owing to the many different fractionation processes employed. However, for potatoes it was shown (Kertesz, 1951) that only 0·07 per cent. (average of 24 varieties) was water soluble compared with the total 4·1 per cent., in keeping with our small loss of soluble pectin on boiling potato disks (Fig. 5).

Nature of the 'water-soluble' fraction. The fraction of the tissue removed by boiling in water for a short time seems to be the same as that removed by NaOH during the saponification process or by the salt solution used during the decalcification method (Table V). From the results of decarboxylation and loss in dry weight it seems probable that the cell contents are being leached from ruptured cells, leaving crude cell-wall material. Different extractants leading to the same ultimate result tend to confirm this view, since if, for example, part of the pectic substances in the cell walls was being degraded by extracting in boiling water, it would be unlikely that cold NaOH and salt solution would have exactly the same effect.

Further work would be required to take account of other substances in the tissues which yield CO_2 on decarboxylation; for example, the occurrence of ascorbic acid and chlorogenic acid in normal tissue has been mentioned but no consideration has been given to the possibility that there might be synthesis of these substances by the tissue during an experiment. Smith (1952) found that disks from selected lots of potato tubers increased in ascorbic acid content by two to three times in 48 hours when incubated at 30° C. in aerated water.

Zucker and Levy (1959) showed that chlorogenic acid formation occurred in disks of potato tuber, inner core tissues, which are initially almost devoid of the compound. Lieberman *et al.* (1959) found that chlorogenic acid increased and ascorbic acid declined during chilling of sweet potato roots at 7·5° C. over a 10 weeks' storage period. These results indicate the complexity of the effects which may be encountered.

The 'water-insoluble' fraction. For the present investigation, the changes in the 'water-insoluble' fraction are of more interest. At the higher temperatures, carrot and the beets show initially significant synthesis of pectic substances, while synthesis by turnip is much less and by potato not at all. These changes can be related to changes in the boiled dry weight. There is little increase in weight by turnip and a steady decrease by potato. It might be, however, that

the vigorous aeration used in these experiments was not entirely suitable for soft tissues such as potato. Experiments at 2° C. showed more variation in replication of results than at higher temperatures and, in general, it can be concluded that there are only small changes taking place. Energy to promote synthesis of pectic substances and a supply of the necessary precursors presumably depend upon the changes in respiration and content of reducing sugars produced by higher temperatures (MacDonald and DeKock, 1958).

It has been shown already (MacDonald, Knight and DeKock, in press) that there is synthesis of both insoluble nitrogenous and phosphorus compounds in carrot and beets, with smaller changes in turnip and very little in potato. These changes account for some of the discrepancies in the increase in boiled dry weight and are also used as evidence of renewed growth by the tissues. Synthesis of pectic substances is accompanied by synthesis of cellulose and hemicellulose (Carlier and Buffel, 1955) which could account for the residual increase in dry weight.

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The Effect of L-Methionine on the Respiration of Plant Tissues

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With three Figures in the Text

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SUMMARY

The respiration of washed disks of storage tissue has been found to be inhibited by L-methionine. The inhibitory effect of methionine appears to be exerted on the Krebs cycle and the inhibition is reversed by dinitrophenol. The possibility that the increased respiration which occurs after washing disks of storage tissue is due to loss of methionine has been raised, and briefly discussed in relation to the tentative suggestion that methionine is a coupling agent between oxidation and phosphorylation.

INTRODUCTION

DURING an investigation into the origin and metabolism of methyl groups in plants, it was observed that L-methionine inhibited the respiration of washed turnip disks and *increased* the specific activity of carbon dioxide produced by disks supplied with glycine–1–^{14}C (Davies and Wilkinson, *in preparation*). These observations led to a study of the effect of methionine on the metabolism of storage tissue.

MATERIALS AND METHODS

Plant material was obtained from the local greengrocer and from the departmental gardens. Disks of storage tissue were cut as described by Sutcliffe (1952) except that the thickness was 1 mm. Disks were washed in aerated distilled water, at room temperature (25° C.) for periods of up to 96 hours. Rates of tissue respiration were determined by measuring the oxygen uptake of twenty disks (fresh weight approximately 1 gm.) suspended in potassium phosphate buffer (2·0 ml., pH 6·0) at 37° C. Carbon dioxide output was not routinely measured, because the respiratory quotient of storage tissue, determined by the direct and indirect methods of Warburg (Umbreit, Burris, and Stauffer, 1949), was found to lie between 0·96 and 1·08 in the presence or absence of methionine. Radioactive carbon dioxide was measured as barium carbonate, which was plated and counted at infinite thinness. The values are expressed as counts per minute per sample and are the average of triplicate samples.

RESULTS

The effect of L-methionine on the rate of respiration of a range of tissues was studied and the results are recorded in Table I.

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TABLE I

Effect of L-Methionine (5×10^{-3} M.) on the rate of oxygen uptake by slices of various tissues

After cutting, tissues were rinsed in distilled water and transferred to Warburg flasks containing potassium phosphate buffer (0·04 M., pH 6·0). Respiration was measured for 1 hour before the addition of methionine.

Plant	Tissue	Per cent. inhibition
Beetroot	root	0
Carrot	root	3
Swede	root	40
Turnip	root	16
Bryophyllum	leaves	22
Horse-chestnut	stem	5
Oat	coleoptiles	3
Pea	epicotyls	30

It is well established that washing disks of storage tissue (Laties, 1957, 1959; MacDonald, 1959) leads to an increased rate of respiration. The effect of methionine on respiration in relation to the period of washing is illustrated in Table II.

TABLE II

Effect of L-methionine on the respiration rate of washed disks of storage tissue

Disks suspended in potassium phosphate buffer (0·04 M., pH 6·0) and respiration measured before and after the addition of methionine (final concentration 5×10^{-3} M.).

Tissue	Washing time (hours)	Respiration µlitres/hr./20 disks	Respiration in presence of methionine (5×10^{-3} M.)	Per cent. inhibition
Beetroot	0	79	79	0
	24	200	175	12
	48	230	150	34
	72	290	180	38
Carrot	0	155	150	3
	24	242	116	52
	48	236	114	52
Swede	0	78	47	40
	24	172	94	46
	48	220	92	58
Turnip	0	75	63	16
	24	148	60	58
	48	190	78	60
	72	200	—	—

Since the inhibition is most pronounced after washing, all subsequent experiments were performed on washed disks.

The effect of buffer concentration on the degree of inhibition of respiration by methionine is shown in Table III.

To study the maximum degree of inhibition and yet maintain adequate control of pH, a buffer concentration between 0·01 and 0·04 M. was used with turnip slices in all subsequent experiments.

The effect of methionine concentration on respiration at a constant buffer concentration is shown in Fig. 1.

TABLE III

Effect of buffer concentration on the inhibition of respiration produced by L-methionine

Methionine concentration 5×10^{-3} M., potassium phosphate buffer pH 6·0.

Tissue	Buffer concentration (M.)	Per cent. inhibition of respiration
Turnip disks washed 72 hours	0	50
	0·008	50
	0·04	48
	0·2	35
Beetroot disks washed 96 hours	0	42
	0·008	30
	0·04	20
	0·2	5

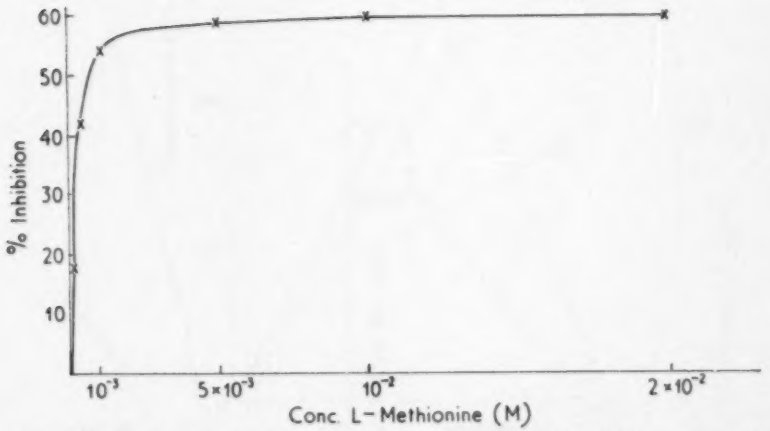

FIG. 1. Effect of methionine concentration on the respiration of turnip disks. Disks washed 72 hours, respiration measured in phosphate buffer pH 6·0, 0·02 M.

The specificity of methionine inhibition was studied by testing the effect of a range of amino-acids, and compounds structurally related to L-methionine, on the respiration of turnip slices (Table IV).

L-methionine was the only compound tested which exerted a significant degree of inhibition.

Since a major component of respiration (60 per cent.) is inhibited by methionine, the effect of methionine on the activity of the Krebs cycle was investigated. Methionine was found to inhibit the production of radioactive carbon dioxide from fumarate-2,3-^{14}C and acetate-2-^{14}C (Table V and Fig. 2). Whilst not conclusive proof, these results support the view that

TABLE IV

Effect of various compounds on the respiration of turnip disks

Disks washed 48 hours. All compounds tested at 5×10^{-3} M. in potassium phosphate buffer (0·02 M., pH 6·0).

Compound	% Inhibition (—) or stimulation (+) of respiration
L-Methionine	−56
D-Methionine	0
L-Methionine sulphoxide	0
methylthiopropylamine	+27
methylcysteine	0
methylcysteine sulphoxide	0
L-alanine	+2
L-glutamate	+5
glycine	+7
L-leucine	+8
L-serine	+5
L-tryptophane	−2

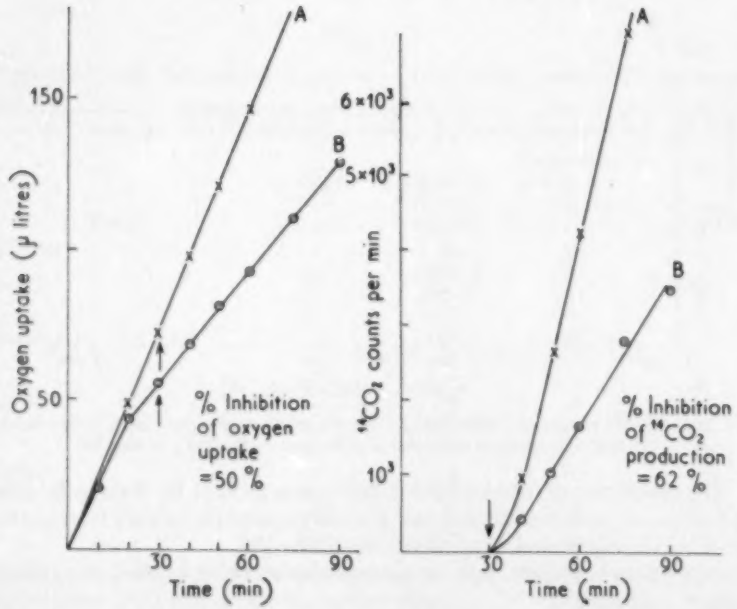

FIG. 2. Effect of methionine (5×10^{-3} M.) on (1) oxygen uptake (2), $^{14}CO_2$ production from acetate–1–^{14}C, by turnip disks. Disks washed 48 hours, respiration measured in phosphate buffer pH 6·0, 0·02 M.

Curve A. Control.

„ B. Methionine added at zero time. After 30 min. acetate–1–^{14}C added to both as indicated by arrows.

methionine inhibits the Krebs cycle. Such inhibition could conceivably be due to a direct blocking of the cycle, in a manner similar to the inhibition of succinic dehydrogenase by malonic acid, or to the inhibition of aconitase by fluorocitrate. Alternatively, the inhibition could be due to methionine inhibiting the

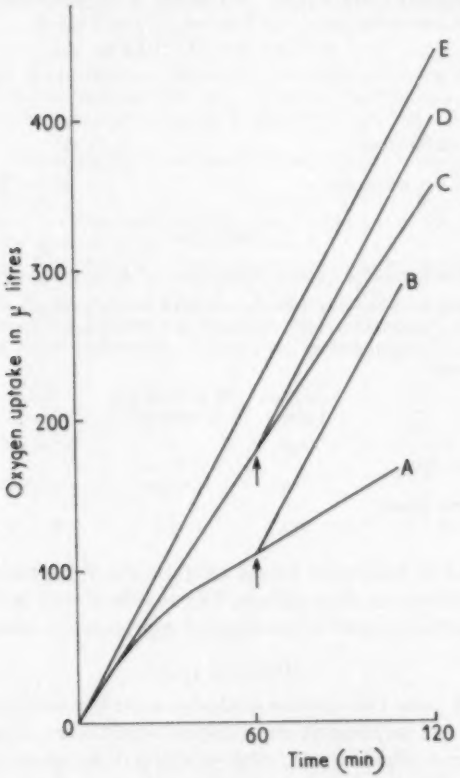

FIG. 3. Effect of methionine (5×10^{-3} M.) and dinitrophenol (DNP 8×10^{-5} M.) on the respiration of turnip disks. Disks washed 48 hours, phosphate buffer pH 6·0, 0·02 M.

Curve A. Methionine added at zero time.
" B. DNP added after 60 min. incubation with methionine.
" C. Control.
" D. DNP added after 60 min. incubation in buffer.
" E. DNP added at zero time.

utilization of adenosine triphosphate, thereby inhibiting that fraction of respiration associated with oxidative phosphorylation. These alternatives can be tested by means of experiments with dinitrophenol which is known to uncouple oxidative phosphorylation. If methionine inhibits respiration by blocking the Krebs cycle, the inhibition should not be relieved by dinitrophenol;

TABLE V

Effect of L-methionine on the oxidation of acetate-1-^{14}C and fumarate-2,3-^{14}C by turnip discs

Each flask contained 20 turnip disks (washed 48 hours) phosphate buffer (pH 6·0, 0·02 M., 2·0 ml.) and acetate-1-^{14}C (1 μMole), fumarate-2,3-^{14}C (1 μMole) and methionine (final concentration 5×10^{-3} M.) as indicated below.

Conditions	$^{14}CO_2$ after 1 hr. as c.p.m.	$^{14}CO_2$ after 2 hrs. as c.p.m.
Acetate-1-^{14}C	23×10^3	$32{\cdot}4 \times 10^3$
Acetate-1-^{14}C + methionine	$3{\cdot}3 \times 10^3$	$12{\cdot}1 \times 10^3$
Fumarate-2,3-^{14}C	$8{\cdot}2 \times 10^3$	$17{\cdot}6 \times 10^3$
Fumarate-2,3-^{14}C + methionine	5×10^3	$11{\cdot}2 \times 10^3$

TABLE VI

Effect of dinitrophenol on the respiration of methionine-inhibited disks

Each flask contained 20 turnip disks (washed 48 hours), phosphate buffer (pH 6·0, 0·02 M., 2·0 ml.), fumarate-2 3-^{14}C (1 μmole 2×10^5 c.p.m.) and additions as indicated in the table. Oxygen uptake expressed as μlitres/hr., $^{14}CO_2$ as counts per min. formed after 1 hour.

Additions	Oxygen uptake	% stimulation or inhibition	$^{14}CO_2$ c.p.m.	% stimulation or inhibition
None	140	—	$2{\cdot}1 \times 10^4$	
Methionine (final conc. 5×10^{-3} M.)	70	−50	$1{\cdot}4 \times 10^4$	−33
Methionine + DNP (final conc. 4×10^{-5} M.)	150	+7	$2{\cdot}6 \times 10^4$	+24

whereas reversal of inhibition would be expected if methionine inhibits the production of adenosine diphosphate. The results shown in Fig. 3 and Table VI demonstrate the reversal of methionine inhibition by dinitrophenol.

DISCUSSION

These results raise two interconnected issues: the mechanism of methionine inhibition and its possible physiological significance. The increase in the rate of respiration which occurs after washing disks of storage tissue could conceivably be due to the removal of methionine. Since the experiments indicate that methionine exerts its effect via oxidative phosphorylation, it is tentatively suggested that methionine acts as a coupling agent. Thus methionine would be expected to maintain the mitochondria of freshly cut tissue in a resting state (Potter and Recknagel, 1951) with a low rate of respiration which is markedly stimulated by dinitrophenol. The removal of methionine by oxidation, decarboxylation, or leaching during the washing of the tissue would lead to some degree of uncoupling, an increased respiration and limited stimulation by dinitrophenol.

Whilst the hypothesis fits the known facts (see the review by Laties, 1957), it would be premature to discuss it further until more experimental results are available.

ACKNOWLEDGEMENTS

It is a great pleasure to record the long sustained encouragement and critical evaluation of Professor T. A. Bennet-Clark, F.R.S., in this and other problems. Thanks are also due to the Royal Society and the Agricultural Research Council for grants towards the cost of chemicals and apparatus.

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The Effect of Anaerobiosis on Acids of the Tricarboxylic Acid Cycle in Peas

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With three Figures in the Text

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SUMMARY

Maturing seeds of the pea (*Pisum sativum*) were subjected to 24 hours' anaerobiosis and then returned to air. Carbon-dioxide evolution was estimated. At intervals samples were analysed for their content of organic acids by silica gel and paper chromatography and for bound carbon dioxide.

During the anaerobic period there was a large accumulation of lactate, an initial increase of succinate, and a slow, continuing decrease of malate and citrate.

On return to air the main changes were a fall in the concentration of lactate and succinate, a rise in malate and acetate, and a rapid rise followed by a fall of pyruvate and α-oxo-glutarate.

Comparison of these changes with each other and with the rate of production of carbon dioxide shows that they do not fit a pattern based on the tricarboxylic acid cycle. The possibility that this was the result of a system of 'pools' of these acids is considered.

INTRODUCTION

THE enzymes required for the oxidation of pyruvate by the tricarboxylic acid cycle (T.C.A.C.) have been shown to be present in particles from a wide range of plants and in mitochondria from most parts of the pea plant (*Pisum sativum*) by Davies (1953 and 1956) and Smillie (1955). There is, however, very little physiological evidence to show the extent to which the system functions as a major respiratory pathway under normal conditions. Turner and Quartley (1956 and 1957) concluded that the major part of respiration of ripening pea seeds was by the T.C.A.C. when they were subjected to a high pressure of oxygen but it does not follow from this that it is equally true of peas in air. On the other hand Beevers and Gibbs (1954) and Gibbs and Beevers (1955) concluded from a study of the rate of liberation of labelled C_1 or C_6 of the glucose molecule during respiration that the maximum percentage of the respiratory carbon dioxide arising from a symmetrical oxidation of the glucose molecule varied from 28–50 per cent. for pea stem internodes and 39–56 per cent. for pea leaves. Further, Mapson and Moustafa (1956) concluded from studies of mitochondria *in vitro* that not more than 50 per cent. of the oxygen uptake of germinating peas was mitochondrial and this strongly suggests that a maximum of 50 per cent. of their respiration was via the T.C.A.C. Thus there is some evidence that in much of the pea plant the T.C.A.C. is of low activity.

The present paper describes the changes occurring in acids of the T.C.A.C. in maturing pea seeds caused by a period of anaerobiosis. The changes described do not give support to the view that the cycle is a major respiratory pathway in this tissue.

MATERIALS AND METHODS

Ripening peas of the variety 'Onward' were used at about 70 per cent. of their maximum fresh weight—the stage previously described as 'mature' (Wager, 1957). Immediately after gathering, the pods were graded for maturity and the peas removed singly and put into successive samples. By this means successive samples always consisted of peas mainly from the same pods and the experimental error within analytical groups was small. At each sampling time separate samples of peas were used for estimation of tissue or bound carbon dioxide (10–12 g.), of oxo-acids (10–12 g.), and of other organic acids (20–24 g.).

The samples of peas, in small glass containers, were put into a water bath at 16° C. and a separate stream of air passed over each group of three samples. After 18 hours the stream of air was replaced by nitrogen gas which was maintained for 24 hours and then the peas were returned to air for a further period of 24 hours. Carbon dioxide emission by some of the samples was measured by passing the gas stream flowing over them through Pettenkoffer tubes containing a solution of barium hydroxide, a continuous record being obtained by the use of a Blackman gas commutator (Matthaei, 1904).

Bound carbon dioxide was liberated by dropping the sample into 20 ml. of $N.H_2SO_4$ at 100° C. and estimated by aerating for 1½ hours through Pettenkoffer tubes, containing barium hydroxide solution.

In 1954 the oxo-acids were extracted after killing the peas in boiling methanol according to the method of Isherwood and Cruickshank (1954). In 1955 the improved method of Isherwood and Niavis (1956) was used, in which the enzymes were inactivated by dropping the peas into methanol and solid carbon dioxide at −70° C. and then destroyed by blending in 5 per cent. metaphosphoric acid at −3° C. In both years the oxo-acids were extracted with successive amounts of 5 per cent. metaphosphoric acid and converted to the 2:4-dinitrophenylhydrazones. After extraction the hydrazones were separated by paper chromatography, using paper buffered with 0·2 M. sodium phosphate at pH 6·25 and run with *tert*-pentanol:ethanol:water (50:5:40) as solvent. The separated hydrazones were eluted with 0·2 M. sodium bicarbonate and estimated by their light absorption at 3,650 Å.

The method of 'hot' killing used in 1954 was tested extensively against the cold killing method of 1955 and was found to give values which were proportionately higher (*c.* 40–60 per cent.) over the whole range of experimental conditions.

The remaining organic acids were estimated by silica gel chromatography (Isherwood, 1946; Wager and Isherwood (in the press)). Lactate and succinate were eluted together from the silica gel columns and were separated

with a different eluent on a second column of silica gel by the method of Neish (1949). During the preliminary purification the lipids were removed by extraction with light petroleum: this also extracted most of the higher fatty acids and these were discarded.

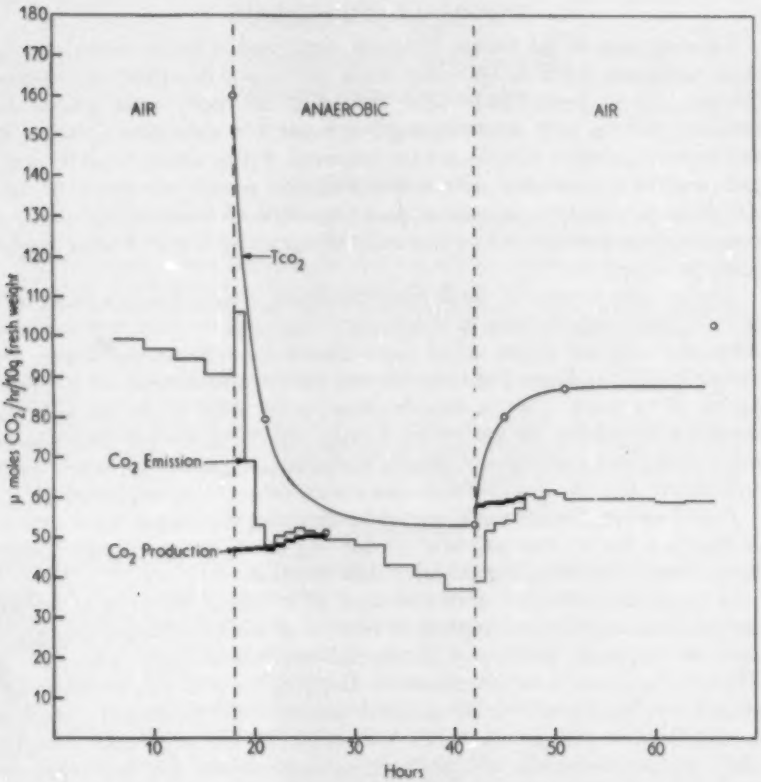

FIG. 1. The rate of carbon dioxide emission, its calculated rate of production, and the concentration of bound carbon dioxide (Tco_2) in maturing peas—first in air, then anaerobic in nitrogen gas, and then in air again. Expt. of 27.7.55.

RESULTS

Oxo-acids have been estimated in a number of experiments in which peas have been kept for a period in air, then transferred to nitrogen, and finally returned to air again. Other acids have been estimated in two such experiments and in the recovery phase of two others. The results of all the experiments are in close quantitative agreement, which suggests that the observed changes show characteristic metabolic patterns for 'Onward' peas and probably for other varieties also.

1. *Evolution and production of carbon dioxide*

Tissue or bound carbon dioxide (Tco_2) decreased when peas were transferred from air to nitrogen (Fig. 1). The change was too large to be explained on the basis of a change in the bicarbonate equilibrium caused by alteration of carbon dioxide pressure and pH, but a discussion of this will not be given in this paper. In any period of time, production of carbon dioxide by the peas must equal that given out (emission) less the change in Tco_2. In most experiments Tco_2 was measured at the end of the initial aerobic period and after 3, 9, and 24 hours of anaerobiosis. The hourly rates of production of carbon

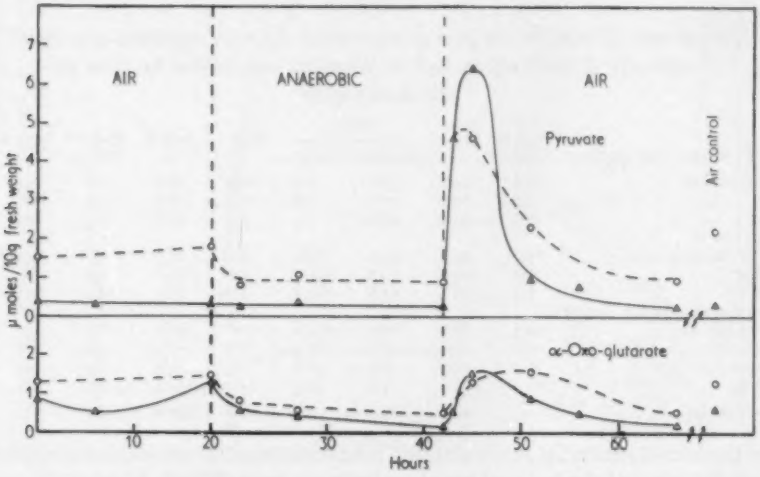

FIG. 2. The concentration of pyruvate and α-oxo-glutarate in peas which were in air, then anaerobic in nitrogen gas, and then in air again.

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——Δ—— Expt. of 27.7.55

dioxide have been calculated on the assumption that change of Tco_2 follows the smoothed curve shown in Fig. 1, an assumption justified by other experiments where Tco_2 was measured hourly. The values so calculated for the production of carbon dioxide are plotted for the experiment of 27.7.55 in Fig. 1, together with the values for emission of carbon dioxide. Similar curves have been obtained in many other experiments so that the shape of the carbon dioxide production curve is well established.

Production of carbon dioxide differed greatly from emission during the first 3–4 hours of anaerobiosis; the rise in emission followed by a slow fall agreed with that calculated for the physical system if it were assumed that the bulk of the bound carbon dioxide was liberated rapidly on becoming anaerobic.

In air after anaerobiosis the rate of production of carbon dioxide was very

nearly constant for the first 9 hours although a slowly falling trend with duration of starvation would be expected. The final value of Tco_2 shown in Fig. 1 is probably in error since there was usually no change in values after 9 hours of air.

2. *The content of organic acids*

The values for the content of pyruvate and of α-oxo-glutarate are plotted in Fig. 2 on a scale ten times as large as that of the other acids in Fig. 3. The concentration of oxalo-acetate in most samples was so low (less than one-tenth

TABLE I

The content of acids in the peas of experiment 27.7.55 expressed as μ equivalents/10 g. fresh wt. in each of the main peaks eluted by silica gel chromatography

Treatment after shelling	Peak 2 Acetate	Peak 3 —	Peak 4 Succinate	Peak 4 Lactate	Peak 5 —	Peak 6 —	Peak 7 Malate	Peak 8 Citrate
0·5 hrs. air	12·6	25·3	12·8	2·6	13·4	18·9	122	253
6 ,,	11·8	21·9	12·6	1·8	19·9	16·4	112	247
18 ,,	10·8	22·1	13·4	1·9	5·3	20·0	145	201
18 hrs. air								
+3 hrs. anaerobic	7·5	24·8	26·0	60·4	5·3	17·3	136	210
+9 ,,	8·4	24·0	30·8	121·0	—	19·5	99	180
+24 ,,	6·9	20·8	33·6	164·0	4·8	21·5	63	151
18 hrs. air + 24 hrs. anaerobic								
+3 hrs. air	20·5	20·6	10·9	121·0	—	20·2	78	151
+9 ,,	24·2	22·4	8·9	75·3	7·3	18·4	99	167
+14 ,,	30·9	23·6	6·4	56·7	—	17·9	89	155
+24 ,,	26·3	22·9	5·2	30·5	11·1	17·4	85	181
66 hrs. air	9·7	20·5	6·0	2·0	8·0	167·0	123	189

of the lowest values for pyruvate) that the estimates obtained were of doubtful significance and the few higher values have not been plotted. In most experiments (28.7.54 is an exception) the concentration of pyruvate did not change during anaerobiosis while α-oxo-glutarate fell. During the recovery in air both acids rose rapidly in concentration and then decreased again.

The chromatographic separation of the organic acids by silica gel distributed them into about 150 fractions which were titrated with 0·01 N NaOH. These titration values, when plotted against fraction number, gave histograms with eight main and four much smaller peaks. As far as could be determined by paper chromatography, peak 1 consisted entirely of fatty acids, peak 2 of acetate, peak 4 of lactate and succinate, peak 7 of malate, peak 8 of citrate, and a much smaller peak 9 of iso-citrate. Peaks 3, 5, and 6 each consisted of several, so far, unidentified acids and will be briefly considered below. The size of the peaks in both experiments was closely similar and values for one only are quoted (Table I). The contents of lactate and succinate were determined by rerunning the acids of peak 4 and these values are given in the table.

The values for the major acids are plotted in Figs. 3A and 3B. The main changes shown in the graphs are a rise in lactate and succinate during anaerobiosis and a fall on return to air; the succinate changes were smaller and of

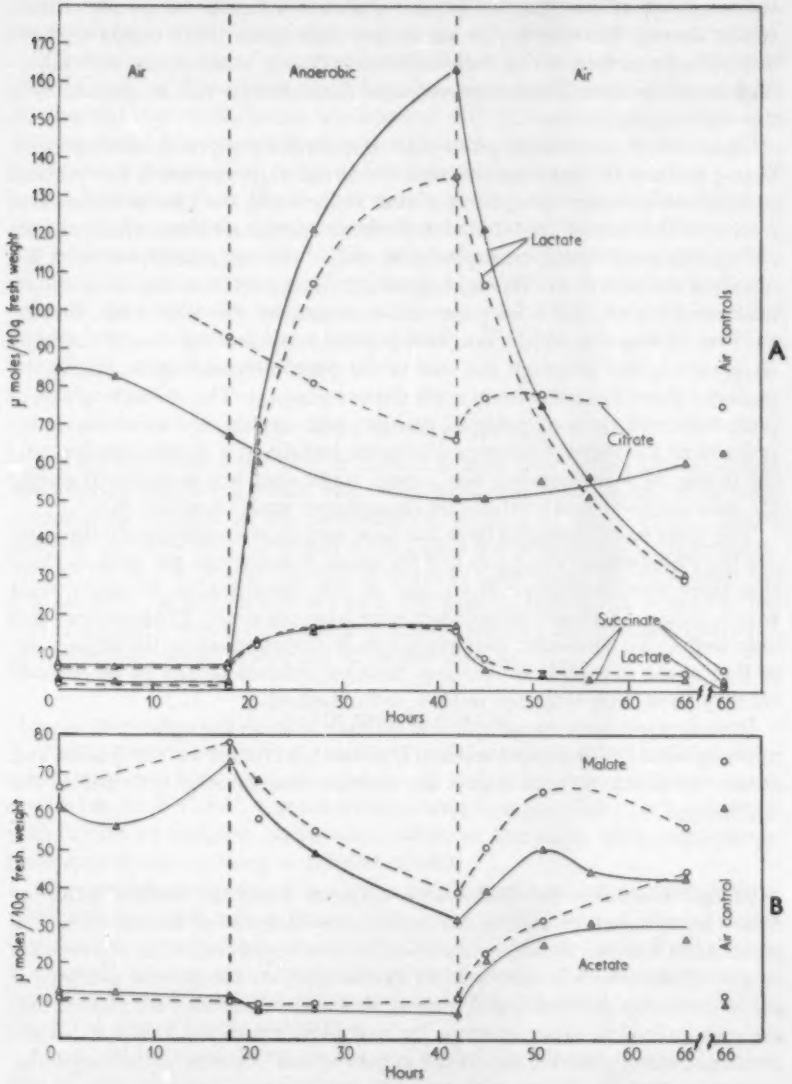

FIG. 3. The organic acid content of peas which were in air, then anaerobic in nitrogen gas, then in air again. (A) Lactate, citrate, and succinate. (B) Malate and acetate.

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shorter duration than those of lactate. Citrate and malate fell during anaerobiosis; during the recovery in air malate rose again while citrate changed little. Finally acetate, which remained steady during anaerobiosis, rose many-fold on return to air. The interpretation of these changes will be considered in the next section.

The acids of the smaller peaks were examined by paper chromatography. Peak 3 is about the expected position of fumarate and contained five acids all of which were shown to be distinct from fumarate by the phenol-formic acid solvent of Stark *et al.* (1951). Although there was no significant change in the size of this peak during an experiment, one of the acids disappeared on the return of the peas to air. The acids present in peak 5 from an initial air sample were similarly separated by paper chromatography into four acids. During the first 18 hours in air the size of this peak decreased and two of the acids disappeared, and although the size of the peak increased again during the recovery phase in air these two acids did not reappear. The chromatograms of peak 6 showed spots agreeing in position with oxalate and *cis*-aconitate together with a number of fainter acidic spots, and the only change noted during the course of an experiment was a large acidic spot which appeared during the later stages of anaerobiosis and disappeared again on return to air.

The acids just considered have not been estimated systematically throughout the whole series of samples and the above observations are given to show that there were a number of changes in the concentration of minor acids which, though small in total amount, were large relatively. These minor acids may well be metabolically important—their concentration is the same order as that of the oxo-acids in air—but their importance cannot be ascertained till they have been extracted in bulk and identified.

In each experiment one sample was maintained in air throughout the experimental period and is plotted separately as the 'air control' on the graphs, and shows the closeness with which the experimental samples returned to the 'air' value.

DISCUSSION

In this discussion the T.C.A.C. is taken to mean the cyclical group of linked reactions which oxidize the carbon of pyruvate to carbon dioxide. The condensing enzyme combines acetyl-CoA, from pyruvate, with oxaloacetate to give citrate which is converted to oxaloacetate via the general pathway of α-oxo-glutarate, succinate, and malate. Reduced nucleotides are formed and are metabolized in other systems. In any given tissue the T.C.A.C., when combined with glycolysis, may be the major route of oxidation of carbohydrate, it may be one of several pathways of carbohydrate oxidation, it may be primarily a method for the formation of intermediates for other systems, or it may not function to a significant extent. One of the objects of the present work is to assess the contribution of the T.C.A.C. to the respiration of the pea. The interpretation of the results is greatly complicated by the possibility that the acids involved in the T.C.A.C. may be metabolized by many other

routes, and especially by transamination of the oxo-acids to the corresponding amino-acids, and that there are 'pools' in the cell, of differing concentrations.

When using results of the type described here to assess the importance of the T.C.A.C. as a respiratory pathway, two main arguments can be used. In the first the rate of respiration is compared with the concentration of possible intermediates, corresponding changes of both being evidence in favour of the operation of the enzyme reactions involving the intermediates. The second argument derives from the cyclical nature of the process being tested, since change in the amount of one intermediate should be balanced by an equal but opposite change in other cycle intermediates. Change in amount of an intermediate with no corresponding change elsewhere in the cycle is evidence, only, of metabolism by pathways extraneous to the T.C.A.C. It is not, of course, evidence against the operation of the T.C.A.C. The results will be considered from these two points of view.

1. *The initial aerobic period in air*

During this period the rate of carbon dioxide production slowly drifted downwards, suggesting steady state conditions; but nevertheless there were considerable changes in the concentration of acids of the T.C.A.C. These changes may have been the result of change in temperature (July air temperature to 16° C.) or of shelling, which alters the gas round the peas from the 2–3 per cent. carbon dioxide found in the pods to that in air. Whatever the cause, the changes in concentration of α-oxo-glutarate and of malate were not related to the rate of production of carbon dioxide.

2. *The anaerobic period*

During anaerobiosis direct oxidation processes (as opposed to intramolecular transfer of hydrogen) must cease and with it *active* metabolism in such systems as the T.C.A.C. A general rise in reduction potential may be expected with a rise in reduced nucleotides, while at the same time high-energy phosphate levels are likely to fall.

Changes in lactate, ethanol, and pyruvate. In these results the most striking effect was the rapid rise in lactate (Fig. 3A): initially the rate of production was about 30 μ moles/10 g./hr. and this fell progressively until after 24 hours it was only 1·5 μ moles. The aerobic production of carbon dioxide was 92 μ moles/10 g./hr. (Fig. 1) and in terms of carbon metabolized was equalled by the initial rate of lactate production.

Comparable in size, but not measured in this experiment, was the production of ethanol. Using results from previous experiments (Wager, 1959) the following mean hourly rates for production of ethanol can be calculated: 0–3 hours, 27 μ moles; 3–9 hours, 33 μ moles; and 9–24 hours, 33 μ moles. Thus the initial rate of ethanol production, also, was about equal to the rate of carbon dioxide production in air but, unlike the production of lactate, changed little with time. This ethanol was equivalent to about 70 per cent.

of the carbon dioxide evolved during the anaerobic period and therefore about 30 per cent. was 'excess' carbon dioxide, i.e. about 15 μ moles/10 g./hr.

Both the ethanol and lactate were presumably formed from pyruvate and their formation is evidence, therefore, of a high ratio of reduced to oxidized nucleotides and of a high rate of glycolysis, since pyruvate, when produced in large amounts, is unlikely to be derived from any source other than sugars (either by the Emden–Meyerhof–Parnas pathway or the pentose phosphate pathway or some other).

At the commencement of anaerobiosis the rate of carbohydrate utilization must have been at least equal to the sum of that used to form lactate (90 μ moles), ethanol (81 μ moles), and an unknown amount, greater than the 15 μ moles per hour emitted, used in the formation of the 'excess' carbon dioxide, i.e. at least 186 μ moles/10 g. per hour. Comparison of this figure with that of carbon dioxide production in air (92 μ moles per hour, Fig. 1) gives the best estimate of the Pasteur effect in these peas; it is many times larger than that deduced only from the rate of carbon dioxide evolution and of ethanol formation (Wager, 1959) and emphasizes the need for comprehensive analysis in the evaluation of the changes in the rate of glycolysis.

In most experiments (28.7.54 is abnormal in this respect) the concentration of pyruvate changed neither during the transition from aerobic to anaerobic conditions nor while anaerobic conditions were maintained (Fig. 2). On the other hand anaerobic conditions must have caused an immediate increase in the utilization of pyruvate (to form lactate and ethanol) and the rate of utilization then slowly fell during the remainder of the anaerobic period. The size of this initial increase in pyruvate utilization depends on the extent to which pyruvate is an intermediate in aerobic respiration; the rise must be at least twofold. The decrease in utilization during the anaerobic period was largely at the expense of lactate formation, which fell from being equal to ethanol formation to about 5 per cent. of it after 24 hours anaerobiosis. Since the equilibrium for the step phospho-enol-pyruvate to pyruvate is very much to the right it is unlikely that the concentration of pyruvate significantly affects its rate of formation, which must be controlled by and equal to the rate of glycolysis. The fall in production of lactate, therefore, must have been a consequence of and be evidence for a fall in the rate of glycolysis, a fall of course not indicated by the carbon dioxide production.

Changes in succinate, malate, and citrate. Succinate accumulated during the early phase of anaerobiosis and malate and citrate fell (Fig. 3). Initially this increase of succinate was roughly balanced by the combined loss of malate and citrate; but the rise in succinate soon ceased, whereas the fall of both malate and citrate continued throughout the period of anaerobiosis. Since most of the loss of malate and citrate was not balanced by the gain in succinate, little significance can be attached to the approximate agreement between the gain and loss during the initial period.

Succinic acid oxidase is normally linked to molecular oxygen via the cytochrome system and therefore the cessation of succinate oxidation under

anaerobic conditions would be expected. If this were so the observed small accumulation of succinate would represent the total movement round the T.C.A.C. under anaerobic conditions. If, however, the succinic acid oxidase system can transfer hydrogen to acceptors other than molecular oxygen *in vivo* the rise in succinate would indicate, only, a slowing of the reaction. A rise in succinate during anaerobiosis has been reported in other tissues (Ranson, 1953).

Most of the considerable loss of citrate and malate that occurred during anaerobiosis was not balanced by gain in any other cycle intermediates and therefore represented a net loss of carbon from the acids intermediate in the T.C.A.C. The results give no indication of how this could have occurred. The most probable routes for loss of malate are conversion to oxalo-acetate and then either transamination to aspartate, or decarboxylation to pyruvate or phospho-enol-pyruvate followed by conversion to ethanol. Citrate might have gone to α-oxo-glutarate and this have been either transaminated or, alternatively, converted to malate, if succinate could have been oxidized anaerobically. Even if both these acids had been ultimately converted to ethanol the carbon dioxide produced would have equalled only a small proportion of the 'excess' carbon dioxide.

3. *The recovery in air*

Changes in lactate. The largest change in acids during the recovery was the fall in lactate (Fig. 3A) which, presumably, was oxidized to pyruvate and therefore is evidence of a sudden and large change in the state of oxidation of the nucleotides. On the assumption that the ratio of DPNH/DPN was constant during the recovery in air the velocity constants for the oxidation of lactate have been calculated for the two experiments:

Time in air	*k* for 27.7.55	*k* for 28.7.54
3 hrs.	0·99	0·76
9 hrs.	0·87	—
14 hrs.	0·67	—
24 hrs.	0·65	0·65

The values from the two experiments agree well and show a slow drift of the constant with time, indicating that lactate concentration was not the only factor concerned. Nevertheless the figures strongly suggest that the loss of lactate was controlled mainly by its concentration and was not greatly affected by the concentration of pyruvate, which rose and then fell again (Fig. 2). It may also be inferred that the ratio of oxidized to reduced nucleotides changed little during this period.

Changes in pyruvate and α-oxo-glutarate. On return to air the concentration of pyruvate rose rapidly to about twenty-five times its previous value and then fell again (Fig. 2). The rate of the T.C.A.C. would be expected to vary with the concentration of pyruvate but in these peas the rate of carbon dioxide production during recovery in air did not change (Fig. 1), and there is therefore a *prima facie* case against the T.C.A.C. being a major pathway of respiration.

This is only vaid if the system involved in the introduction of pyruvate into the T.C.A.C. was not saturated under normal conditions and if the increase in pyruvate was in contact with this system, i.e. if there were either no pools of pyruvate or the increase in concentration was in the 'active' one. Direct evidence about 'pools' of pyruvate in the pea is lacking but the probability of a small concentrated pool (e.g. in the mitochondria) is not high, because the glycolytic enzymes are in the non-particulate cytoplasm and glycolytic pyruvate is presumably formed there and must diffuse into the mitochondria. The location of lactic dehydrogenase in the cell is unknown but it is presumably cytoplasmic (i.e. not in an isolated part of the cell) and pyruvate formed by it should have been as available to the T.C.A.C. as that from glycolysis.

If the pyruvate were generally distributed throughout the cytoplasm, as seems to be probable from the foregoing considerations, the possibility that the enzymes utilizing it were saturated seems to be small because it was present in such a very low concentration, only 20 μM. when the peas were in air, which would be very low to fully saturate an enzyme, and may be compared with a 'K_M' for pyruvate uptake by mitochondria *in vitro* of 300 μM. (Montgomery *et al.*, 1956).

There are thus some reasons for assuming that the changes in concentration of pyruvate during the recovery should have influenced the rate of the T.C.A.C. if it were a major metabolic route, and until the contrary has been found it seems best to accept the results as evidence against the T.C.A.C. being the major pathway of the respiration of these peas.

It seems probable, therefore, that the rapid increase in the concentration of pyruvate was a result of the rapid oxidation of lactate, and the subsequent fall was caused by a slowing of the production from lactate combined with a gradual utilization by many sluggish systems, presumably including the T.C.A.C.

α-oxo-glutarate shows a marked rise and fall in concentration during the recovery phase, which occurred a little after that of pyruvate (Fig. 2). In the absence of inhibition of the α-oxo-glutarate oxidizing system this should cause an increase in throughput of the T.C.A.C. Since there was no change in the rate of production of carbon dioxide this also is a *prima facie* argument against the presence of an active T.C.A.C., but this conclusion also is liable to alteration if 'pools' can be shown to be present. An alternative reason for the rise in α-oxo-glutarate is that its concentration is linked to that of pyruvate via transamination reactions.

Changes in succinate, citrate, malate, and acetate. On return of the peas to air the concentration of succinate fell rapidly to its level before anaerobiosis (Fig. 3A). This occurred while the concentration of its presumed precursor, α-oxo-glutarate, was high. Furthermore, the fall in succinate cannot have been linked to the considerable rise in malate (Fig. 3B) which also occurred during the recovery, since the rise in malate continued long after the fall in succinate had ceased. The succinate change, therefore, does not fit into a pattern of change based on the T.C.A.C.

Malate (Fig. 3B) showed a considerable rise in concentration, and the carbon in it must have come from outside the T.C.A.C.; the most probable source is synthesis from phospho-enol-pyruvate or pyruvate, and carbon dioxide (cf. dark fixation reactions of Crassulacean plants—Bradbeer *et al.*, 1958). There is no reason to expect this increase in concentration to have affected the rate of the T.C.A.C. because the level of malate was as high as that generally used to obtain maximum rate of pyruvate oxidation by mitochondria *in vitro*; furthermore, Montgomery and Webb (1956) with heart mitochondria obtained a 'K_M' for malate of 0·05 mM. when pyruvate was present in excess, whereas the change in peas here considered is from 2–3 mM.

There was also a considerable rise in acetate during the recovery (Fig. 3B). The rate of acetate accumulation was roughly proportional to the concentration of pyruvate and may have arisen from it by oxidation.

CONCLUSIONS

The foregoing discussion has shown that the rate of production of carbon dioxide by peas was not related to the concentration of intermediates of the T.C.A.C. nor were the changes in amount of intermediates such as would be expected if they were in a cyclical process (cf. p. 41). Thus the results do not support the view that the T.C.A.C. was a *major* pathway of respiration in these peas. It cannot, however, be concluded in consequence that the T.C.A.C. was *not* a major pathway for several reasons: e.g. the rate of the cycle may be controlled by co-factors and not by the concentration of the main substrates, there may be 'pools' of reactants in the cells not in equilibrium with the enzymes of the T.C.A.C., changes in amounts of substrates may be the result of metabolism extraneous to the T.C.A.C., &c. Since large changes occurred in the concentration of many of the acids, they were clearly not storage products isolated from active cell metabolism; furthermore, the new production of acids must have been in the cytoplasm and not isolated from enzymes. Thus there is some evidence against a rigid pool system.

If the rate of the cycle were controlled entirely by co-factors, results of the type described here would show very little; but such an extreme situation seems unlikely, and if there is a balance of factors controlling the enzyme rates the alteration in concentration of the acid substrates should have had an effect on the rate of the T.C.A.C.

The experiments give physiological evidence of slow but considerable metabolism of plant acids in response to change in conditions (shelling, anaerobiosis). The metabolic pathways concerned cannot, in general, be defined but they probably involve the T.C.A.C. itself as well as many routes which are extraneous to it, since much of the carbon metabolized in the changes either enters or leaves the combined 'pool' of acid intermediates of the T.C.A.C. One such route, and a very active one, has been clearly shown, namely the reversible formation of lactate from pyruvate. There is also clear evidence of large changes in the rate of glycolysis with physiological conditions, changes not reflected in carbon dioxide emission.

ACKNOWLEDGEMENTS

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Effect of Monuron and of Related Compounds on the Growth of Seedling Peas

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With three Figures in the Text

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SUMMARY

Shoots of peas grown in phosphate buffer were no more sensitive to monuron applied continuously than were roots. They were, however, more readily damaged than roots when high monuron concentrations were applied for short periods of time. Neither root nor shoot was more sensitive to monuron in light than in darkness. The damage caused by a high monuron concentration was far greater at times, or under conditions, of high metabolic activity. The toxicities of alkyl or aryl substituted ureas and thioureas were closely related to their solubilities in water. In view of this similarity with physical poisons and the probable sites of their main toxic actions, it is considered more likely that they disrupt an integrating surface than that they act upon specific active groups of enzymes.

INTRODUCTION

WESSELS and Van der Veen (1956) demonstrated that the photolytic reaction in isolated chloroplasts is suppressed by concentrations of substituted ureas and carbamates lower than those affecting cell division. The primary action of monuron (3-(*p*-chlorophenyl)-1,1-dimethylurea) is, therefore, usually regarded as an effect on the photolytic side of photosynthesis, although the concentrations suppressing the Hill reaction are not herbicidal to the intact plant. Moreland and Davis (1956) observed that urea and carbamate derivatives show an inverse relationship between solubility and their ability to inhibit an amylase. The same relationship has frequently been observed for physical poisons, for which no specific interaction with enzymes has yet been demonstrated. Such specificity seems excluded by the range of substances and variety of toxic effects involved. Gavaudan and Brebion (1945) found that benzene and chlorobenzene, which are typical physical poisons, also affect cell division and photosynthesis. Similarly, several of the carbamates are well established as narcotics, while others are herbicides which suppress the Hill reaction and have a c-mitotic effect.

The objects of this paper are to show that the inhibition of growth produced by monuron is not an indirect effect due to its action on photolysis and that growth-inhibiting power is related to solubility for a wide range of substituted ureas and thioureas.

EXPERIMENTAL METHODS

Satisfactory growth of untreated peas was obtained by growing them in aerated shallow water at pH 6·4–6·8. Water-saturated air was bubbled into solutions in Pyrex crystallizing dishes, diameter 12 cm. An aeration rate of approximately 100 ml. per dish per minute gave healthy growth. Each dish contained 190 ml. distilled water, or distilled water plus toxicant, and 10 ml. M/15 phosphate buffer, pH 6·75, were added to each to give a total solids content, excluding toxicant, of about 500 p.p.m.

The pea variety 'Improved Pilot' which was used at first proved rather prone to bacterial infection, and was abandoned in favour of 'Meteor'. Comparative tests using several urea derivatives, including monuron, showed that the pea variety used made little difference. Peas were soaked in running shallow water until the radicles were 1–2 cm. long. Then 10 or more were removed to each crystallizing dish and the toxicant applied. By this means the peas with deformed roots could be rejected. After suitable periods of time, the effects of the toxicants were assessed by measuring the length of the shoot, main root, and of the three longest secondary roots, of each seedling. Thus, for secondary roots, 30 readings were averaged at each concentration. Complete statistical analysis was not attempted, since errors in solubility measurements were probably larger than those of a biometric nature. Either monuron or nitrourea was used as a standard throughout the comparative tests, and the susceptibility of the peas varied very little. Median inhibitory concentrations (LD_{50}) were estimated after fitting probit lines by observation.

Most experiments with monuron, and all with other substituted ureas and thioureas, were carried out at 19±2° C. in dull light. The low-temperature tests with monuron were at 8° C. in a refrigerator provided with a low wattage bulb, and were aerated. In one series of tests, the toxicity of monuron on peas grown in bright sunlight was compared with that in total darkness.

Solubilities of toxic substances were found by heating excess solid with distilled water at 60° C., followed by shaking at room temperature for 12 hrs. before filtering. Evaporation was carried out in a vacuum desiccator. Replicates on different solutions of one substance differed little except, rarely, for the least-soluble substances. Dissociation constants of selected compounds were either determined by potentiometric titration, or shown by this method to be sufficiently small to ensure negligible ionization at the pH employed in the tests. The latter was true of all substances listed in Table III. Starch was determined after hydrolysis to glucose by the method of Hanes (1929).

RESULTS

Effects of monuron applied continuously and intermittently

Figure 1 shows the effect of monuron on the growth of the shoot. Secondary roots responded similarly, the median inhibitory doses after 24 days being 25 p.p.m. for roots and 27 p.p.m. for shoots. A four- or five-fold change in concentration alters the effect from one which is very small to one of almost

complete suppression of growth. The combined effects of soil type and rainfall can readily account for a change in effective concentration of this magnitude under field conditions.

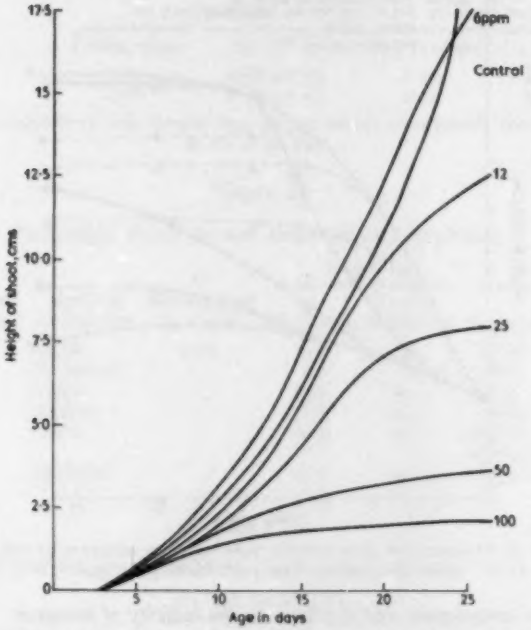

FIG. 1. Effect of different concentrations of monuron on growth of the shoot. (Concentrations in p.p.m. Probit slope at 24 days, 2·0. Pea variety 'Improved Pilot'. pH 6·75.)

The effect on the main root of removing seedlings to water after different times of contact with monuron is shown in Fig. 2. Three days' contact with 100 p.p.m. monuron causes no permanent injury, but for contact periods longer than this the retardation of growth cannot be fully overcome. Injury to the shoot is severe and occurs sooner, while subsequent growth of secondary roots is normal if the peas are removed from monuron before the root initials are clearly visible externally. In other trials, the peas were grown in water, and subsequently removed to 50 p.p.m. monuron for varying times during the first 10 days of growth. After contact periods of 12 hrs. there was no permanent damage to any organ. However, any period of 28 hrs. contact between the 3rd and 10th days caused inhibition of shoot growth almost as great as that due to the same concentration applied continuously from the time the peas were placed in monuron. Main roots were not affected. Secondary roots are as readily inhibited as the shoot for a few days after their first appearance, but are much less sensitive both before and after this period. In

general, the shoot is, therefore, damaged much more readily than roots by intermittent applications of high monuron concentrations.

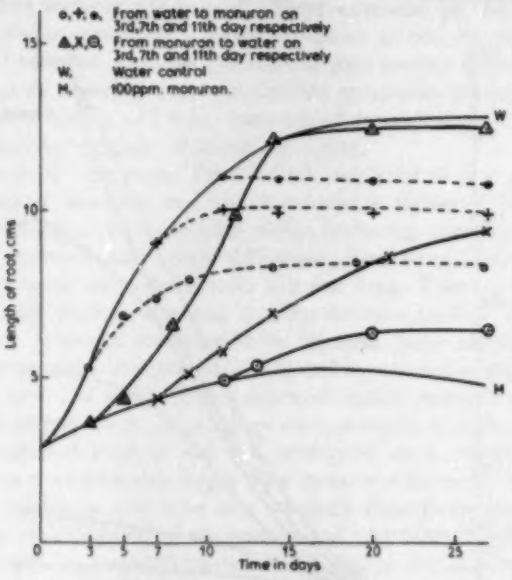

FIG. 2. Growth of the main root after transfer from water to monuron or from monuron to water. Pea variety 'Improved Pilot'. pH 6·75.

Effects of low temperature and darkness on the toxicity of monuron

In contrast to the 28-hr. period of contact with monuron which at room temperature was sufficient to ensure irreversible suppression of the growth of the shoot and emerging secondary roots, much longer contact periods had little effect when the metabolic rate of the organisms was reduced. Peas with secondary roots 1–5 mm. long were transferred to 50 p.p.m. monuron at 8° C. for 5 days. Subsequent growth of roots in water at room temperature was as great as that of peas temporarily in water at 8° C. Growth in each case was delayed by a time equivalent to the refrigeration period.

The effect of light was studied by growing peas in varying concentrations of monuron in strong light and in darkness. Results in Table I show that no significant difference in the sensitivity of secondary roots of peas grown under the two conditions could be detected for periods up to 20 days. The shoot responded similarly. Since photosynthetic conditions did not influence the growth-retarding effect of monuron, the cotyledons of peas grown in some of the dishes were analysed for hydrolysable carbohydrate (Table II). Evidently monuron neither materially alters the rate of mobilization of starch nor requires food reserves to be depleted before the inhibitory action on growth can begin.

TABLE I

Effect of monuron on secondary roots of peas grown in direct sunlight and in total darkness

	10 days contact		20 days contact	
	Probit slope	$m \pm SE_m$	Probit slope	$m \pm SE_m$
In light	1·77	0·88±0·14	2·50	1·07±0·17
In darkness	1·77	0·99±0·10	2·50	1·24±0·08

m, log median inhibitory dose; probit lines do not depart significantly from parallelism at 10 or 20 days.

TABLE II

Percentage starch on wet weight of pea cotyledons

Concentration of monuron	Lighting condition	At germination % starch	10 days		20 days	
			% starch	% root inhibition	% starch	% root inhibition
None	Light	25·4	14·9	0	11·0	0
	Darkness	..	14·0	0	10·0	0
10 p.p.m.	Light	..	13·7	64	11·2	61
	Darkness	..	13·9	64	11·4	51
50 p.p.m.	Light	..	14·5	93	(disintegrating)	96
	Darkness	..	13·7	93	13·0	90

TABLE III

Toxicities and solubilities of some alkyl and aryl ureas

Substance	5+Log molar LD_{50}		Relative saturation	Solubility g./l.
	Var. Pilot	Var. Meteor		
Urea	1·942	2·669	..	500
Sym-dimethyl urea	3·146	3·152	..	∞
Ethyl urea	3·456	..	..	∞
Phenyl urea	1·882	1·871	0·017	6·11
m-tolyl urea	..	1·489	0·016	2·93
Fenuron	..	0·874	0·004	2·90
Monuron	0·914	0·843	0·063	0·23
2-naphthyl urea	..	0·664	0·093	0·09
Sym-diphenyl urea	0·501	..	0·056	0·12
Thiourea	..	2·298	0·0017	49·5
Ethyl thiourea	..	1·983	0·0020	50·6
Sym-diethyl thiourea	..	1·816	0·0023	38·5
Dimethylol thiourea	1·117	..	0·0099	1·80
Phenyl thiourea	..	1·062	0·0080	2·19
m-tolyl thiourea	..	0·752	0·0040	2·34
Di-(n-butyl) thiourea	0·561	0·370	0·020	0·29
Di-xenyl thiourea	..	Satd.	1·0	0·02
Ethyl carbamate	..	3·571	0·0034	1000
phenyl urethan	1·358	..	0·055	0·69
iso-propyl phenyl carbamate	..	0·160	0·083	0·032

LD_{50}, median inhibitory dose.

Secondary root inhibition by substituted ureas, thioureas, and carbamates

Toxicity and solubility data of alkyl and aryl derivatives of urea, thiourea and carbamic acid appear in Table III. The relative saturation is the toxic concentration (for 50 per cent. inhibition of growth) divided by the saturation concentration in water. The median inhibitory doses (LD_{50}) recorded are the average values from two or more independent tests and, where both varieties of pea were employed, the relative saturation is the average for the two. In Fig. 3 the log molar toxic concentrations are plotted against log molar solubilities. The line for thiourea derivatives was fitted by the method of least squares, that of substituted ureas being drawn parallel to it. The thioureas are thus about twice as toxic as urea compounds when the solubility factor is kept constant. While specific chemical effects may play some part in determining overall toxicity (e.g. the aberrant position of fenuron, 3-phenyl-1,1-dimethyl urea) it is apparent from Fig. 3 that for substances in the same series, phase distribution plays a predominant role.

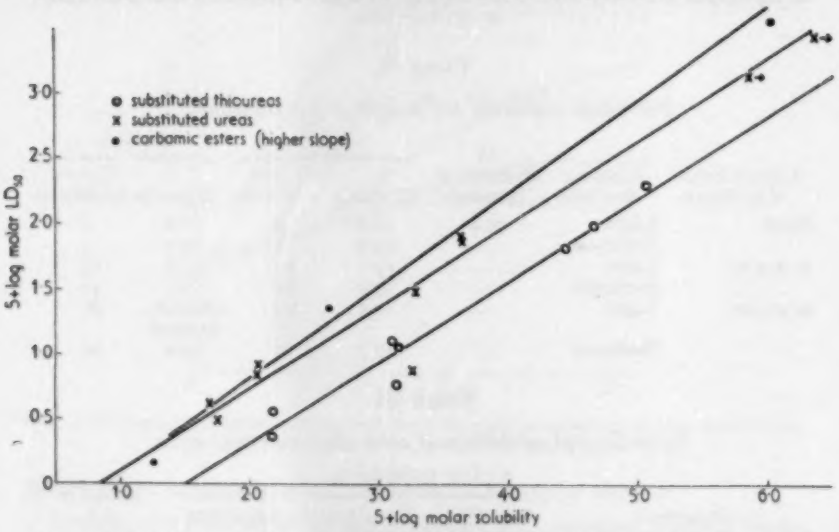

FIG. 3. Toxicity to secondary roots of some substituted ureas, thioureas, and carbamates, as a function of solubility in water.

The toxicities of compounds other than alkyl and aryl substituted ureas and carbamates appear in Table IV. No relationship with solubility is apparent and the isotoxic relative saturations vary widely. Nitrourea and hydrazine nitrourea are of interest in view of their high toxicity. According to Davis and Blanchard (1929), nitrourea de-arranges to give cyanic acid. The toxicity of potassium cyanate is included in Table IV, for Lees and Simpson (1957) consider that cyanate is the actual toxicant when nitrourea inhibits the oxidation of nitrite by *Nitrobacter*. The lower toxicity of the highly dissociated

TABLE IV

Toxicities of ureas substituted with acidic or basic groups, and of some related compounds

Substance	5+log molar LD_{50}		Relative saturation	Solubility g./l.
	Var. Pilot	Var. Meteor		
Nitrourea . . .	0·545	0·559	17×10^{-3}	21·7
Hydrazine nitrourea . .	..	0·372	15×10^{-6}	219
Potassium cyanate . .	..	1·426	46×10^{-6}	474
Guanyl urea sulphate .	0·523	0·242	0·0003–5	32
Semicarbazide hydrochloride	..	1·124	0·00004	383
Asym-acetylphenyl urea .	0·378	0·279	0·018	0·22
p-chloro-acetanilide . .	0·201	0·764	0·013–0·048	0·20
2-chloroaceto-acetanilide .	0·260	0·443	0·005	0·96

cyanate could be due to the site of de-arrangement of the nitrourea and the degree of dissociation of the molecular species entering the plant, but it is worthy of note that the pK_A value of nitrourea is about 4·0, which is consistent with 99·8 per cent. of the nitrourea being in the ionized state at pH 6·7.

DISCUSSION

Using growth inhibition of pea seedlings as a measure of toxicity, the root and shoot are affected equally by a given concentration of monuron. This differs from certain experiments on mature plants, where the leaves or shoot have been reported to be damaged at concentrations innocuous to roots. However, in the present tests the shoot was readily injured by temporary applications of high concentrations of monuron. In seedlings, and possibly also in mature plants, this apparently greater effect on the shoot could be due to accumulation in the shoot as a result of transpiration. Illumination tests show that light does not increase the susceptibility of the root or shoot to monuron during the 20-day period for which observations were made, while subsequent analyses indicate that growth is retarded long before food reserves in the cotyledons are used up (Tables I and II). Jasmin and Ferguson (1953), Christoph and Fisk (1954–5), and Tysset, Vachet, and Mathieu (1958) all report cell division to be affected by monuron. Growth retardation in seedlings, and hence under pre-emergence spray conditions in the field, could well be a direct consequence of this effect. Intermittent application of monuron indicates that the time when irreversible damage occurs most rapidly coincides with the most active period of growth of the organ concerned, while refrigeration tests show that long contact periods with high monuron concentrations have little effect when the metabolic rate is low.

An approximate comparison with field application is possible if it is assumed that monuron, which is sparingly soluble, remains in the top 0·1–0·4 in. of soil for some days. 1 lb./acre would then correspond to 40–160 p.p.m. in aqueous

solution, and selective herbicidal action at the usual application rate could be readily accounted for as a direct action on growth.

The correlation between solubility and toxicity shown in Table III and Fig. 3 is typical of physical poisons and occurs when iso-molar concentrations at a non-aqueous site of action are of approximately equal toxicity. While slow diffusion through some intermediate physical barrier can sometimes have a similar practical result, Haun and Peterson (1953) have shown that monuron enters plants readily and even accumulates in the shoot. Mayer (1956) similarly demonstrated a very rapid entry of thiourea. A general relationship of a similar sort can be found in the results of Wessels and Van der Veen (1956) and of Moreland and Davis (1956), in isolated systems where conventional diffusion barriers do not exist.

Since all the substituted ureas and thioureas in Table III act within a limited range of relative saturation, the growth-retarding effect of monuron cannot be attributed to some special stereochemical feature of its molecule. The toxic effects of this and other ureides are consequently not likely to require high specificity of the type proposed by Wessels and Van der Veen (1956), even if the same enzymes could be presumed to be present at several different sites of action. It is worth recalling that the toxicities of urethanes quoted by Rabinowitch (1945) also show the homologous series effect characteristic of physical poisons, the concentrations affecting photosynthesis being about one-third of those affecting respiration.

A physical poison has been described by Albert (1958) as 'merely a diluent separating biochemical components which should react together'. Using this definition, it is suggested that the compounds in Table III are physical poisons, and that the different toxic effects shown by them in living organisms have one factor in common, namely, that whether the site of action is the chloroplast, the mitochondrion or the cell membrane, they act upon systems involving lipoprotein phase boundaries.

While a fuller understanding of the action of ureides and carbamates must await progress in structural biochemistry, it is desirable that attention should be drawn to the possibility that quite a range of substances may affect enzymic processes, not by a specific attachment to the enzyme, but by distortion of an organizing surface at which integrated reactions occur.

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Effects of Illuminance near and below Light Saturation on the Time Course of Photosynthesis in Synchronized Cultures of Algae[1]

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With five Figures in the Text

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SUMMARY

The effect of light intensity on the rate of the photosynthetic gas exchange was studied in synchronized suspensions of the high-temperature strain, Chlorella 7-11-05. It was found that, contrary to previous investigations, even the light intensity far below light saturation can be harmful to the photosynthetic activity of algal cells. The ability of cells to withstand the inhibitory effects of light and to use increasing light intensity efficiently depends largely on the developmental status of the cells. Studies on the effects of light on non-synchronized populations may produce misleading results because statistical averages of the illuminance characteristics of cells of different developmental stages may cancel and largely obscure the inherent physiological characteristics of the individual cells.

INTRODUCTION

THE time course of the gas exchange during a photosynthesis experiment has been described for non-synchronized algal suspensions either by more or less constant, falling, or increasing rates (Sorokin, 1960*a*). A decreasing rate has usually been ascribed to inhibitory effects of some external factors or to a deficiency of the medium in some essential nutrient salts (Emerson, 1935). An increasing rate has generally been attributed to growth phenomena allegedly taking place during photosynthetic measurements (Emerson, 1935). A rate constant over a portion of the photosynthetic experiment has commonly been taken as an indication of a 'steady-state'.

Deductions have been made also in the opposite direction—from the shape of the time-course curve investigators have deduced in what degree conditions during observations were favourable for the photosynthetic process. This reasoning has been universally accepted, particularly in studies of the inhibitory effects of strong light. A decline of the time-course curve of photosynthesis rate has commonly been accepted as an indication of harmful effects of a given light intensity.

Studies on synchronized algal populations have indicated that the time course of the photosynthetic gas exchange greatly depends on the developmental status of cells (Sorokin, 1960*a*). The effects of light, like those of other

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environmental factors, superimpose on the basic developmental characteristics of the individual cells.

MATERIAL AND METHODS

The work was done with the high-temperature strain, Chlorella 7-11-05. Characteristics of the organism, and the techniques of growing cells and of

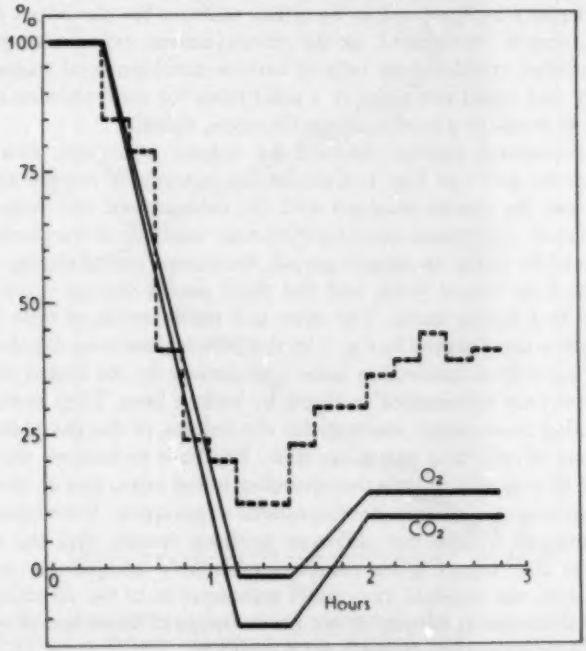

FIG. 1. Time course of photosynthetic gas exchange in 6-hour cells of *C. pyrenoidosa*, strain 7-11-05, at 1,900 foot-candles. Manometric readings corrected for the gas volume and the amount of cells in manometric flasks are given by broken lines. The results, in terms of the exchange in O_2 and CO_2, obtained by the conventional two-vessel technique during the three periods of one photosynthetic experiment are represented by solid horizontal lines, the thin oblique lines serving as connecting links. The rates of gas exchange are expressed in percentages of the initial rates at the start of the experiment.

measuring their photosynthetic gas exchange have been described in previous publications (Sorokin, 1959 and 1960*a*). The source of light during photosynthesis measurements was a bank of tungsten lamps placed under the glass bottom of the bath. Variations in light intensity were obtained with neutral filters attached to the bottoms of the reaction vessels. The results of measurements of photosynthetic activity are given as changes in the volume of the gas phase in the manometric flasks per volume of packed cells per 10-minute

intervals. No corrections for respiration were made and the data therefore represent an apparent photosynthetic gas exchange.

Interpretation of the experimental results in terms of a particular gas—carbon dioxide or oxygen—was avoided for the following reason. Studies were made with the one-vessel technique. Calculation of the results in terms of the exchange in oxygen or in carbon dioxide would involve assumption of an assimilatory quotient. Although assimilatory quotients for cells of different developmental stages proved to be rather uniform for the initial portion of each manometric experiment, as the photosynthetic experiment progressed the assimilatory quotients for cells of various developmental stages changed drastically and could not serve as a solid basis for computations of the gas exchange in terms of a particular gas (Sorokin, 1960*a*).

The time-course curves obtained for 6-hour cells with two different techniques are given in Fig. 1. Calculations in terms of oxygen and carbon dioxide from the results obtained with the conventional two-vessel method (Umbreit *et al.*, 1957) were made for three time intervals of one photosynthetic experiment: the initial 20-minute period, the second period during which the rate reached its lowest level, and the third period during which the rate recovered to a higher level. The rates and the intervals of time for which they are given are depicted in Fig. 1 by thick horizontal lines, the thin oblique lines serving only as connecting links. Calculations by the technique adopted in this report are represented in Fig. 1 by broken lines. They were obtained by correcting manometric readings for the volume of the gas phase and for the amount of cells in a particular flask. For both techniques the rates are expressed in per cent. of the corresponding initial rates, that is, the rates for the first 20 minutes of each photosynthetic experiment. Examination of the curves obtained by the two different methods reveals that the technique adopted in this report gives results qualitatively comparable with those obtained with the standard two-vessel technique as to the direction, extent, and time of changes in the rate of the gas exchange in the course of one photosynthetic experiment (cf. Sorokin 1960 *a* and *b*).

RESULTS

Photosynthetic activity in 0- and 8-hour cells was studied at comparable light intensities—410 foot-candles for 0-hour and 470 foot-candles for 8-hour cells (Fig. 2). The illuminance of 400 foot-candles is well below light saturation, which for both kinds of cells was found to be close to 1,500 foot-candles (Sorokin, 1960*c*). In 0-hour cells the rate of the gas exchange increased constantly during more than 3 hours of observations. Since the suspending fluid consisted of only 0·02 M. of potassium dihydrogen phosphate, there was no nitrogen uptake during observations. Growth, if it has taken place in the course of measurements, must therefore occur at the expense of nitrogen present in 0-hour cells at the beginning of the experiment. Under the same illuminance and in the same suspending fluid the rate of gas exchange in 8-hour cells decreased steadily during about 4 hours of observations. Explanation

of the decrease in the rate in the phosphate buffer as due to injurious effects of the phosphate solution (Emerson, 1935) seems unsupportable, since no injury was recorded in 0-hour cells. The decrease in photosynthetic activity in 8-hour cells could be accounted for by the lack of essential salts in the phosphate buffer (Emerson, 1935) if one assumed that the 0-hour cells were

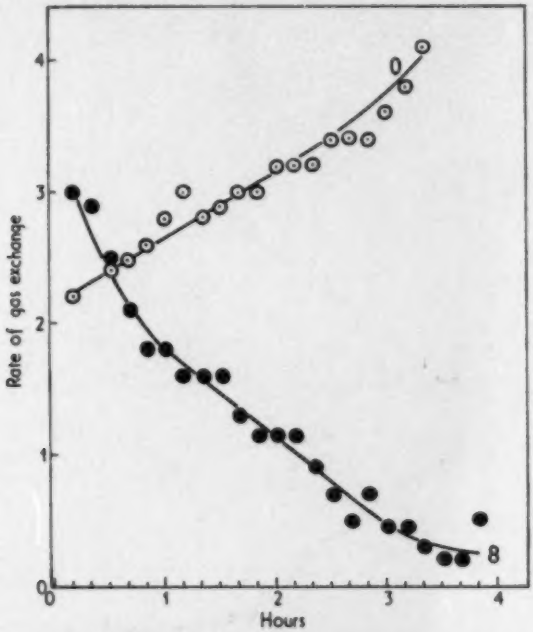

FIG. 2. Rates of gas exchange in the course of a photosynthetic experiment in 0- and 8-hour cells of *C. pyrenoidosa*, strain 7-11-05, at light intensities close to 400 foot-candles. Increments in manometric readings (mm. pressure change) for 10-minute intervals were corrected for the volume of the gas phase and the amount of cells in manometric flasks. The developmental stages of cells are indicated in hours on the curves. Light intensities: for 0-hour cells, 410 foot-candles; for 8-hour cells, 470 foot-candles.

capable of storing these essential salts in amounts sufficient to support a continuous increase in photosynthetic activity in these cells for more than 3 hours of observations.

Photosynthetic activity in 0-, 2-, 4-, 6-, and 8-hour cells at light intensities close to 800 foot-candles is described in Fig. 3. Light saturation for 4- and 6-hour cells is at 1,700 foot-candles and for 2-hour cells is above 2,200 foot-candles (Sorokin, 1960c). The course of the gas exchange for 0- and 8-hour cells at 800 foot-candles (Fig. 3) resembles that at 400 foot-candles (Fig. 2). However, the difference in the performance of the two kinds of cells is accentuated at higher light intensities. The slopes of the curves and the

differences between rates at the start of the experiments and those after 3 hours of observations are greater at 800 than at 400 foot-candles. For 2- and 4-hour cells the course of photosynthetic activity is essentially similar to that for 0-hour cells. As had been established previously (Sorokin, 1960*a*), the activity in 2-hour cells increased faster and reached a higher level after 3 hours of observations than that in either younger or older cells. In 6-hour cells the

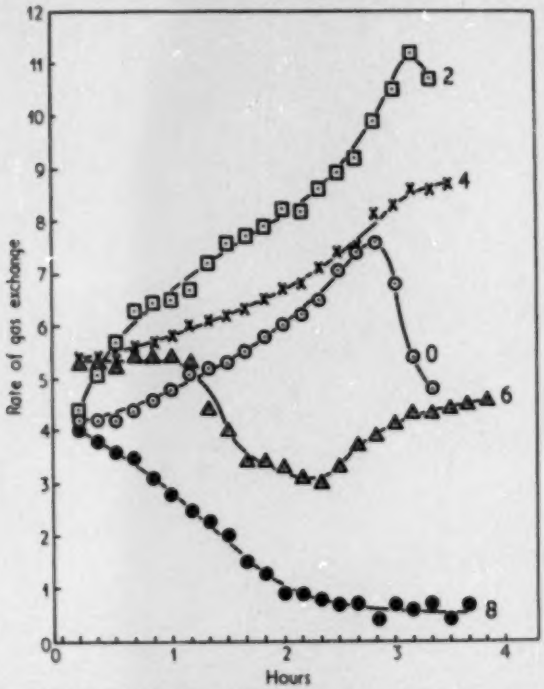

FIG. 3. Rates of gas exchange in the course of a photosynthetic experiment in cells of successive developmental stages of *C. pyrenoidosa*, strain 7-11-05, at light intensities close to 800 foot-candles. Increments in manometric readings (mm. pressure change) for 10-minute intervals were corrected for the volume of the gas phase and the amount of cells in manometric flasks. The developmental stages of cells are indicated in hours on the curves. Light intensities for 0-, 2-, 4-, 6-, and 8-hour cells, respectively, 790, 810, 820, 780, and 760 foot-candles.

phenomenon reported in a previous publication (Sorokin, 1960 *a* and *b*) is clearly discernible: the rate of the gas exchange after some time starts to decline, reaches a low point, and then recovers to a higher level.

At light intensities near 1,500 foot-candles (Fig. 4) the time-course curves for 0- and 8-hour cells are only slightly different from those at lower light intensity. For 0-hour cells the increase in photosynthetic activity continues for a shorter time than at lower light intensity. In 8-hour cells, after a period

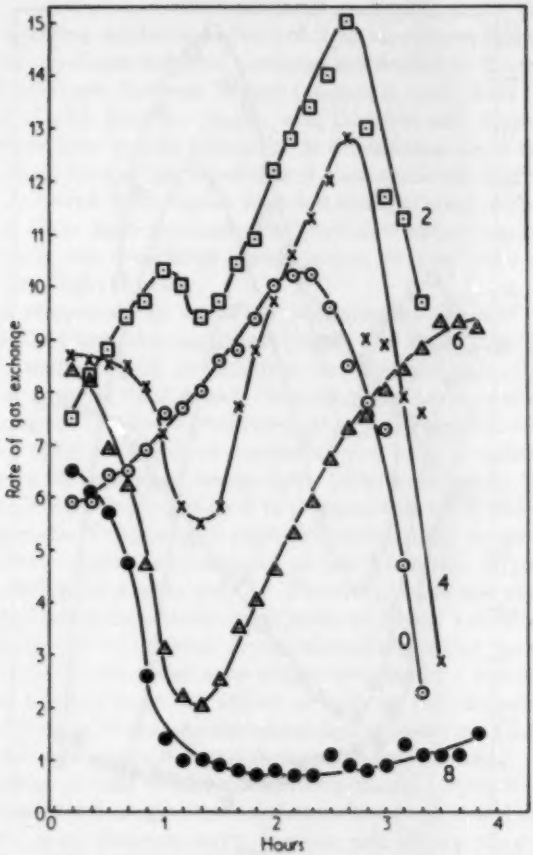

FIG. 4. Rates of gas exchange in the course of a photosynthetic experiment in cells of successive developmental stages of *C. pyrenoidosa*, strain 7-11-05, at light intensities close to 1,500 foot-candles. Increments in manometric readings (mm. pressure change) for 10-minute intervals were corrected for the volume of the gas phase and the amount of cells in manometric flasks. The developmental stages of cells are indicated in hours on the curves. Light intensities for 0-, 2-, 4-, 6-, and 8-hour cells, respectively, 1,510, 1,540, 1,560, 1,500, and 1,470 foot-candles.

of about 3 hours, there is a slight increase in the rate of gas exchange. Therefore, at higher light intensity a very weak tendency for a recovery of the photosynthetic rate was observable even in 8-hour cells. In cells of intermediate age the phenomenon of the fluctuating rate is clearly recognizable. The decrease in the rate starts earlier and continues to a lower level in 6-hour than in 4-hour cells. In 2-hour cells the tendency of the photosynthetic rate to go down with time is so weak that it produces only a small dent on the otherwise continuously rising curve of the gas exchange. As the result, the rates

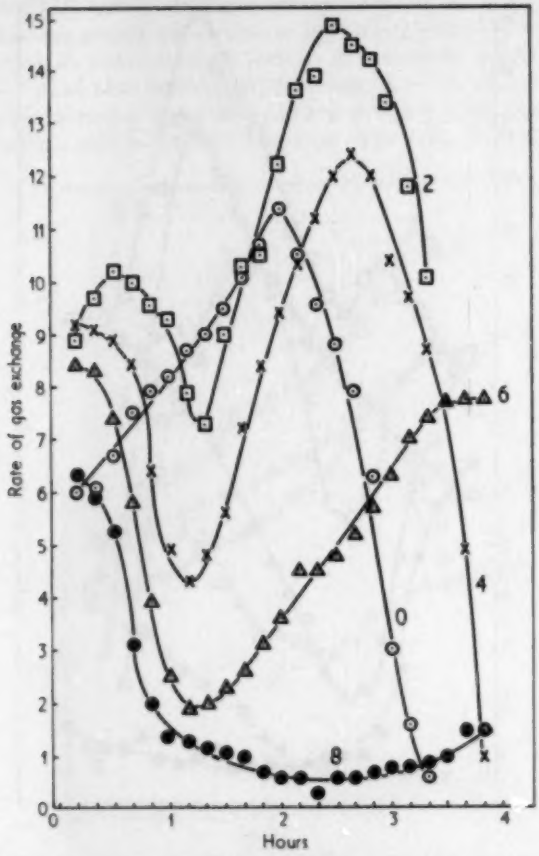

FIG. 5. Rates of gas exchange in the course of a photosynthetic experiment in cells of successive developmental stages of *C. pyrenoidosa*, strain 7-11-05, at light intensities close to 2,100 foot-candles. Increments in manometric readings (mm. pressure change) for 10-minute intervals were corrected for the volume of the gas phase and the amount of cells in manometric flasks. The developmental stages of cells are indicated in hours on the curves. Light intensities for 0-, 2-, 4-, 6-, and 8-hour cells, respectively, 2,110, 2,030, 2,050, 2,160, and 2,100 foot-candles.

during the recovery period reach a higher level than in cells of earlier or later developmental stages.

The light intensity of 2,100 foot-candles lies above light saturation of photosynthesis in all the cells studied except 2-hour cells, for which light saturation was not achieved. The general dependence of the rate of the gas exchange during a photosynthetic experiment on the developmental status of cells was well demonstrated also at this light intensity (Fig. 5).

DISCUSSION

Evidence regarding deleterious effects of light on photosynthesis has been sought by many investigators. Most pertinent are studies by Emerson (1935), Myers and Burr (1940), Steeman Nielsen (1949 and 1952), Burk (1953), Kok (1956), Sironval and Kandler (1958), and Kandler and Sironval (1959). Inhibitory effects were usually looked for at illuminance many times higher than that necessary for the light saturation of photosynthesis. Light intensities approaching or several times higher than full sunlight were often employed. Apparently, at lower light intensities the inhibitory effects were not found, evidence of them was considered unconvincing, or they were ascribed to factors other than light intensity.

Recovery of photosynthetic rates from the injurious effects of strong light has been noticed by several investigators (Myers and Burr, 1940; Kok, 1956; Sironval and Kandler, 1958). Invariably it was observed only after the light was reduced or replaced by darkness. Commonly the recovery was seen only after a lapse of time. The time factor involved might suggest that basic to this recovery was a different degree of susceptibility of cells of various developmental stages to the effects of strong light. Older cells might be killed by exposure to such light or injured by it to a degree that made them incapable of resuming growth. Younger cells might be injured little or not at all. The observed recovery might simply depend on the proportion of younger cells and on their ability to resume growth. Therefore, what was reported as a recovery of photosynthetic activity might really be largely a result of selective effects of light on cells of different developmental stages and the subsequent growth only of those cells which were able to withstand the injury.

The studies referred to on the effects of light on the photosynthetic gas exchange were made with non-synchronized algal suspensions. Characteristics of a non-synchronized microbial population represent statistical averages for the characteristics of cells of different developmental stages. Since cells of various developmental stages have different levels of metabolic activity (Tamiya *et al.*, 1953; Sorokin, 1957; Sorokin and Myers, 1954 and 1957), statistical averages for cellular characteristics obtained from studies of non-synchronized populations may have little physiological significance.

After the amplitude of variability among the individual cells had been narrowed by synchronization technique, the effects of light were shown to depend greatly on the developmental status of cells. Even such a low light intensity as one-fourth of that necessary for light saturation can be detrimental for cells approaching the end of the life cycle. In younger cells the same and higher light intensities favour increase in photosynthetic activity. In cells of intermediate age, both detrimental and favourable effects of the same light intensity are observable during the same photosynthetic experiment and without any change in experimental conditions.

Two probable reactions, one involved in the decrease in photosynthetic activity in the course of a photosynthetic experiment and another responsible for the recovery of the photosynthetic rate, have been discussed elsewhere

(Sorokin, 1960*a*). Their possible connexion with the turnover of chlorophyll molecules and their general dependence on the processes basic to cell growth and cell development have been also subjects of speculation (Sorokin, 1960*c*). The finding that factors connected with cell development have a role greater than has previously been attributed to them in affecting the reactions of living matter to external conditions may require reconsideration of several concepts and ideas entertained in the field of kinetics of photosynthesis and other cellular activities.

ACKNOWLEDGEMENT

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The Effect of High Concentrations of Auxin on the Extension of *Avena* Coleoptile Sections

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With five Figures in the Text

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SUMMARY

The response of *Avena* coleoptile sections to high concentrations of auxin has been determined in the absence of all additives except sucrose. In most experiments the growth-time curves with 75 p.p.m. IAA showed two linear phases. In the first phase, which lasted for only 2–4 hours, extension was as rapid with 75 p.p.m. IAA as with 5 p.p.m. IAA. This rapid initial expansion phase was then succeeded by a second phase which persisted for at least 20 hours. During this second linear phase the growth-rate with 75 p.p.m. IAA was lower than with an auxin concentration of 5 p.p.m. In some experiments the first phase was absent and only the second phase was present.

The response of sections to high concentrations of auxin was not influenced by the presence of buffers or absorbable cations. Omission of sucrose or the presence of moderate amounts of ethanol caused the resulting growth curves to be non-linear.

The rate of uptake of auxin into the tissues was dependent on the auxin concentration and was constant for at least 24 hours.

INTRODUCTION

THERE has been considerable controversy in recent years concerning the effect of high concentrations of auxin on the growth-rate of *Avena* coleoptile sections. Bonner (1933) found that as the concentration of auxin was increased, the growth-rate attained a maximum at an auxin concentration of about 5 p.p.m. indoleacetic acid (IAA) and then decreased as the amount of auxin was increased. At all concentrations of auxin up to at least 100 p.p.m. the growth-rate was found to be constant during the first 16 hours (Bonner and Foster, 1955). Other investigators found apparently conflicting results, however. Bennet-Clark and Kefford (1954), using different experimental conditions, reported that for the first few hours after auxin application high concentrations of auxin caused sections to grow more rapidly than did optimal concentrations of auxin, but that subsequently the growth-rate of these sections progressively decreased, often becoming negative.

It seemed that all the results fell into one of three groups, depending upon the conditions employed. When the growth curves were determined in the presence of sucrose, buffer and potassium ions, growth curves which were linear for the first 16 hours were obtained for all concentrations of auxin (Bonner and Foster, 1955). If buffer and potassium ions were omitted, the

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presence of superoptimal amounts of auxin caused the growth curves to become non-linear (Bennet-Clark and Kefford, 1954). In the absence of sucrose, growth curves were non-linear for all concentrations of auxin (Bentley and Housley, 1953; Housley *et al.*, 1954; Marinos, 1957; Hancock, 1959).

A question arose as to which response of the tissue to superoptimal concentrations of auxin was a response to auxin itself and in which cases was it an artifact due to the experimental procedure exployed. Bennet-Clark and Kefford (1954) suggested that the constant growth-rates obtained with high auxin concentrations were an artifact caused by the presence of absorbable cations. Bonner and Foster (1955) proposed that the non-linearity of the growth curves found in the absence of buffers and potassium ions was due to the adverse effects of pH on the tissues. The present investigation was undertaken in an attempt to settle the question.

EXPERIMENTAL METHODS

Avena seeds were germinated in vermiculite which had been soaked in tap water. The plants were grown in a cabinet in which the temperature was kept at 25° ± 2° C. and the humidity at 90 ± 10 per cent. After 72 hours in the dark, the plants were illuminated with a weak red light to reduce mesocotyl growth. Coleoptiles of 2·5–3·0 cm. in length were then selected (*c.* 96 hours) and after the apical 3 mm. had been discarded, the next 5 mm. of coleoptile was removed as one section. This operation, and all subsequent operations, were carried out under a weak red light. The sections were randomized by placing them in distilled water until all were cut. One lot of 20 sections was measured by means of a microscope fitted with an eyepiece micrometer and the remaining sections were placed in the experimental solutions. Each solution consisted of 20 ml. of 1 per cent. sucrose solution containing IAA and other additions as indicated. All solutions were made up in glass-distilled water; IAA was dissolved in warm water and a fresh solution was made for each experiment. After varying lengths of time, lots of 20 sections were removed from each solution and measured. Growth–time curves were then constructed.

EXPERIMENTAL RESULTS

The effect of superoptimal concentrations of IAA (75 p.p.m.) on the growth of *Avena* coleoptile sections was determined in the absence of all additives except sucrose. In the majority of the experiments (14 out of 19) the response could be divided into two distinct periods. For the first 2–4 hours the sections treated with 75 p.p.m. IAA underwent a rapid initial expansion; they expanded slightly more rapidly than did the sections which were treated with 5 p.p.m. IAA and which acted as controls. But then the growth-rate of these sections suddenly dropped to a value less than that of the controls and remained constant for the remainder of the experimental period (24–30 hours). This can be seen in Fig. 1.

This response to high concentrations of auxin is not affected by the presence

of either absorbable cations or buffers. When sections were incubated in standard 1 per cent. sucrose plus 75 p.p.m. IAA solutions with or without further addition of an absorbable cation (0·01 M. KCl) or a buffer (0·0025 M. K-maleate, pH 4·8), the resulting growth curves were identical for all three treatments (Fig. 2, Table I).

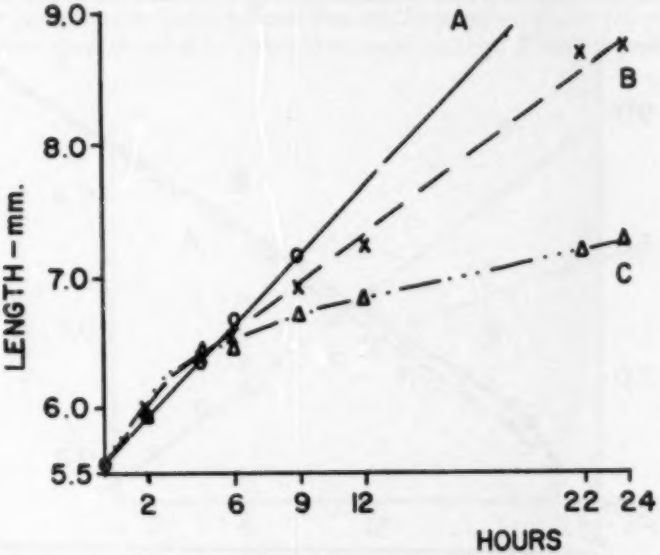

FIG. 1. Response of *Avena* coleoptile sections to superoptimal concentrations of IAA. Demonstration of the rapid initial expansion which occurs in the majority of the experiments and of the effect of sucrose upon the growth curves. Sections treated with 1% sucrose and 5 p.p.m. IAA (A), 1% sucrose and 75 p.p.m. IAA (B), or 75 p.p.m. IAA (C).

TABLE I

The effect of certain additives to the media and changes in the procedure upon the response of Avena *coleoptile sections to high concentrations of auxin*

Modification of procedure	Presence of rapid initial expansion	Subsequent growth-rate linear
None	yes	yes
Sucrose omitted	yes	no
+ 0·1% ethanol	yes	no
+ 0·0067% ethanol	yes	yes
+ 0·0025 M. K-maleate	yes	yes
+ 0·01 M. KCl	yes	yes
+ 0·001 M. EDTA	yes	yes
+ 3×10^{-4} M. KCN	no	no
+ cold (0° C.)	no	no
Seedlings grown in distilled water	yes	yes
Sections pretreated 5 hours with 10 p.p.m. IAA	yes	yes

This response of sections to high concentrations of auxin is different from all previously described types of response. The rapid initial expansion has been reported by Bentley and Housley (1953), Bennet-Clark and Kefford (1954), Housley *et al.* (1954), and Marinos (1957). All of these investigators found, however, that after the rapid initial expansion, the growth-rate did not drop to a constant value but continued to decrease until growth had ceased. Why did the

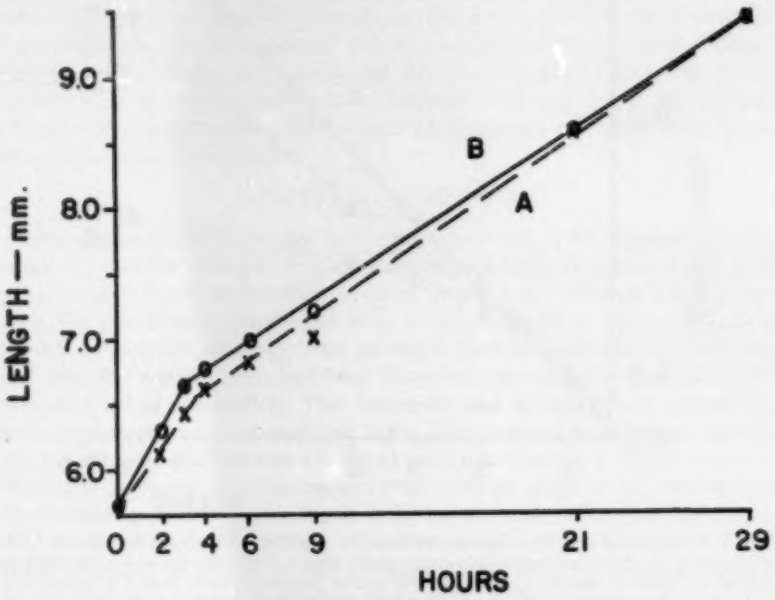

FIG. 2. The effect of buffers and absorbable cations upon the response of sections to super-optimal concentrations of IAA. Sections incubated in 1% sucrose, 75 p.p.m. IAA solutions in the presence (A) or absence (B) of 0·0025 M. K-maleate (pH 4·8).

growth-rate continually decrease in these cases and not in the present experiments? In the experiments of Bentley and Housley (1953), Housley *et al.* (1954), Marinos (1957), and Hancock (1959) the non-linearity of the growth curves may be due, at least in part, to the lack of sugars in the experimental media. Sugars promote the expansion of *Avena* coleoptile sections (Thimann and Schneider, 1938) both by allowing osmoregulation to occur (Ordin *et al.*, 1956) and by providing substances which can be metabolized to give the energy necessary for expansion. When growth curves are determined in the absence of sugars, they are non-linear at all auxin concentrations (Bentley and Housley, 1953; Hancock, 1959). In the present experiments, if sucrose was omitted from the media, the growth-rates continually decreased (Fig. 1). However, the lack of sucrose does not explain the fact that in the experiments

of Bennet-Clark and Kefford, Bentley and Housley, and Marinos the growth-rate fell to a negative value after application of superoptimal concentrations of auxin. The sections appeared to have been injured by the treatment. The extreme sensitivity of the sections to only moderately high concentrations of auxin (less than 100 p.p.m.) may simply be a result of the manner in which the seedlings were grown and the sections were collected in these experiments. If this were true, it would indicate that seedlings grown under the present conditions were much more resistant to auxin toxicity. A second possibility

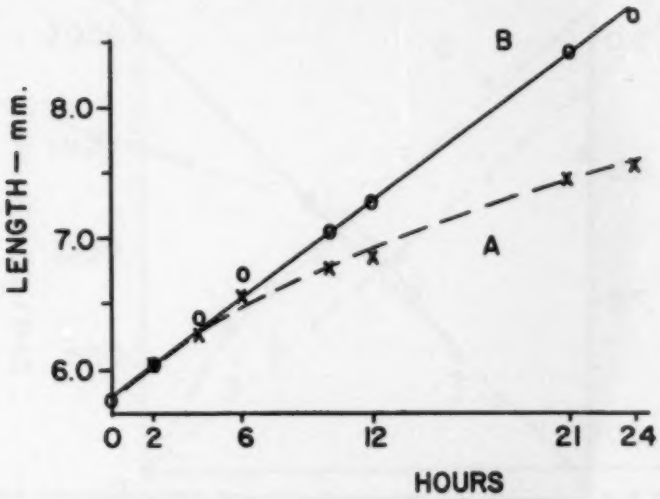

FIG. 3. The effect of ethanol on the response of sections to superoptimal concentrations of auxin. Sections incubated in 1% sucrose, 75 p.p.m. IAA solutions in the presence (A) or absence (B) of 0·1% ethanol.

is that in some of the previous investigations the sections were suffering from ethanol toxicity. IAA is often brought into solution by being dissolved in a little ethanol. If this were done, a stock solution which had an auxin concentration of 750 p.p.m. could easily contain 1 per cent. ethanol. Dilution of this stock to give a final auxin concentration of 5 p.p.m. would give an ethanol concentration of 0·0067 per cent., a concentration which has no effect upon the growth rate. But dilution to only 75 p.p.m. IAA would give an ethanol concentration of 0·1 per cent. and this concentration has been shown to cause the growth-rate of sections treated with 75 p.p.m. IAA to fall off rapidly although it did not cause toxicity in these experiments (Fig. 3).

A series of experiments was undertaken in order to gain some knowledge about the nature of the rapid initial expansion. Although the rapid initial expansion was found in most experiments, it was not an inevitable part of the response of sections to high concentrations of auxin. In 5 of 19 experiments,

the growth-rate of the sections treated with 75 p.p.m. IAA was constant during the whole of the experimental period and was less than that of the controls. These results are identical with those of Bonner and Foster (1955).

The rapid initial expansion cannot be due to a purely physical effect upon the cell wall of the high concentration of auxin, for metabolism seems to be necessary for it to manifest itself. This was shown by incubating sections with 75 p.p.m. IAA either at 2° C. or in the presence of 3×10^{-4} M. KCN.

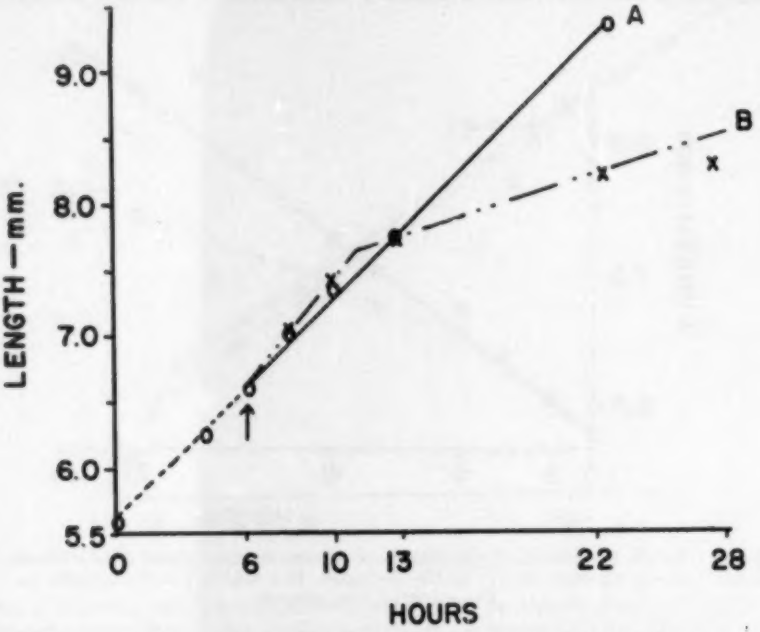

FIG. 4. Demonstration that the duration of the rapid initial expansion is independent of the time elapsed from cutting of the sections. Sections incubated for 6 hrs. in 5 p.p.m. IAA + 1% sucrose and then placed (at arrow) in 1% sucrose solutions containing 5 p.p.m. IAA (A) or 75 p.p.m. IAA (B). Compare with Fig. 1.

Both of these conditions block the effect of 5 p.p.m. IAA on expansion (Cleland and Bonner, 1956). Under these conditions the sections expanded at the same rate as did non-auxin-treated control sections (Table I).

A rapid initial expansion has never been found in experiments conducted in the laboratory of Dr. Bonner, while it has been found in the majority of the present experiments. The most conspicuous difference in the experimental technique used in the two laboratories is that seedlings are grown in distilled water in Pasadena and in tap water in the present case. It was thought that some impurity in the tap water might account for the differences. To test this possibility, seedlings were grown in vermiculite which had been washed three

times with glass-distilled water and then the effect of 75 p.p.m. IAA on the growth of sections taken from these plants was compared with that of sections taken from plants grown in vermiculite washed with tap water. The growth curves were identical in both cases, indicating that the differences cannot be attributed to the tap water (Table I). Addition of 0·001 M. EDTA to the incubation media likewise produced no effect upon the curves (Table I).

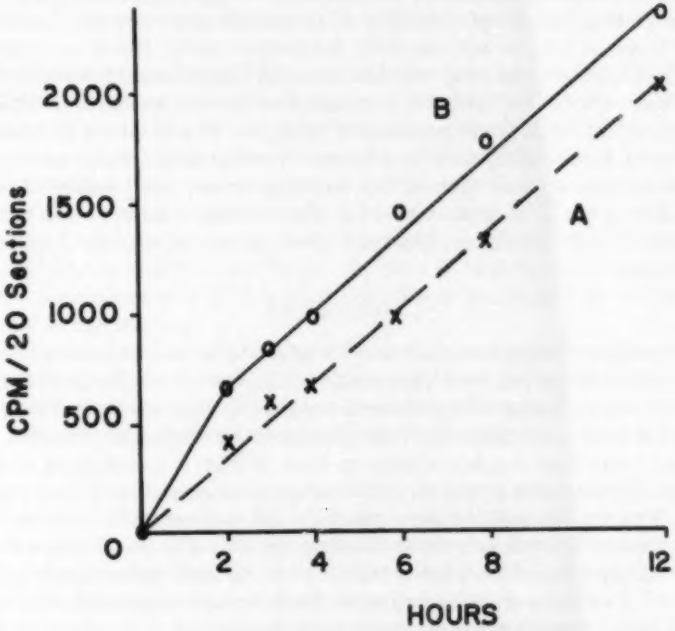

FIG. 5. The effect of auxin concentration upon the uptake of auxin into *Avena* coleoptile tissue. Sections incubated in 1% sucrose, 2 p.p.m. IAA–^{14}C solutions which contained either 3 p.p.m. (A) or 73 p.p.m. (B) unlabelled IAA.

When 75 p.p.m. IAA was added to sections immediately after cutting, the rapid initial expansion persisted for not more than 4 hours. The length of time to which it persisted was not related to the time which had elapsed since cutting. This was shown by the fact that when sections were allowed to expand for 6 hours in a 5 p.p.m. IAA solution and were then transferred to a solution containing 75 p.p.m. IAA, a rapid expansion which persisted for 2–4 hours still occurred (Fig. 4).

Blackman *et al.* (1959) and Bennet-Clark and Wheeler (1959) have found evidence to indicate that when *Lemna minor* and potato tissues are in contact with high concentrations of auxin there is a rapid uptake of auxin for the first 2 hours followed by a loss of auxin from the tissue. The uptake of 2,4–D

by *Avena* coleoptile tissues has been examined by Johnson and Bonner (1956), but since they employed a maximum incubation time of only 2 hours, their results do not show whether such a process occurs in this tissue or not. No such process occurs in the *Avena* coleoptile under the present experimental conditions. This was shown as follows. Sections (5 mm. with the central leaf removed) were incubated with 5 or 75 p.p.m. IAA. Two p.p.m. of the IAA in each case consisted of carboxyl labelled IAA–^{14}C (specific activity 30 μC/mg.). After varying lengths of time, lots of 20 sections were removed, cut in half lengthwise to remove solution from the central cavity, placed on a planchet and dried. The radioactivity was then counted. The results are shown in Fig. 5. The rate of uptake of IAA was constant after the first two hours with both 5 and 75 p.p.m. IAA. In the presence of 75 p.p.m. IAA there was an additional uptake of auxin during the first 2 hours. Growth measurements taken on the same sections showed that in this experiment the rapid initial expansion lasted for 4 hours. It appears that the effect of auxin concentration upon the uptake of IAA into the sections as a whole cannot explain the rapid initial expansion.

DISCUSSION

The effect of high concentrations of auxin upon the expansion of *Avena* coleoptile sections has been determined in the absence of all additives except sucrose. In a majority of experiments the growth–time curves with 75 p.p.m. IAA show two approximately linear phases. In the first phase, which persists for not more than 2–4 hours after addition of high concentrations of auxin, the growth-rate with 75 p.p.m. IAA is as great or greater than with 5 p.p.m. IAA. This rapid initial expansion phase is then succeeded by a second phase which persists for at least 20 hours. During this second linear phase the growth-rate with 75 p.p.m. IAA is lower than with an optimal auxin concentration of 5 p.p.m. In a minority of experiments the first phase was absent; the second phase began immediately upon addition of auxin.

A striking feature of the present results is that the growth-rate of sections treated with superoptimal concentrations of auxin always remained constant during the whole of each phase of growth. The constancy of the growth-rate required the presence of sucrose but was not influenced by the presence or absence of buffers or absorbable cations. This suggests that the linearity of the growth–time curves obtained by Bonner and Foster (1955) was not an artifact caused by the presence of absorbable cations.

Such linearity has not always been obtained. Bentley and Housley (1953), Housley *et al.* (1954), Bennet-Clark and Kefford (1954), Marinos (1957) and Hancock (1959) have all found that the growth-rate of sections teated with similar levels of auxin continually decreased after the first 2–4 hours until all expansion had ceased. In many cases the sections actually shrank due to some toxic effect of the experimental procedures. The present results indicate that the non-linearity cannot be ascribed to a lack of buffering of the system. It is more likely that it is due to one or more of the following possibilities.

The growth-rate of a section will continually decrease during expansion if osmoregulation does not occur, for as the contents of the vacuole become diluted during expansion, turgor pressure will decrease. In the experiments of Bentley and Housley, Housley *et al.*, Marinos, and Hancock, expansion was determined in the absence of any substance which could provide material for osmoregulation. Under such circumstances growth-rates would be expected to fall at all auxin concentrations. In the present experiments the presence of sucrose allowed osmoregulation to occur during expansion (Ordin *et al.*, 1956).

The lack of osmoregulation will not explain the toxicity symptoms shown by the sections in the experiments of Bennet-Clark and Kefford, Bentley and Housley, or Marinos. In some of these experiments sections may have been suffering from ethanol toxicity. In at least some of the experiments of Bennet-Clark and Kefford, IAA was brought into solution by being dissolved in a little ethanol; the same procedure may have been used by Marinos and by Bentley and Housley who do not state the method used to bring IAA into solution. If IAA is dissolved in ethanol, the ratio of IAA to ethanol is fixed. When the concentration of IAA is high, the ethanol level could be sufficient to be toxic to the sections.

A third and perhaps more likely possibility is that the methods used to grow *Avena* seedlings by these investigators has resulted in plants which are extremely sensitive to damage by high concentrations of auxin. The non-linear growth-rates may simply be a reflection of the sensitivity of these sections to high levels of auxin.

A second striking feature of these results is the presence of a period of rapid initial expansion which persists for 2–4 hours after addition of high levels of auxin. A rapid initial expansion of similar duration has been previously reported by Bentley and Housley, Bennet-Clark and Kefford, Housley *et al.*, and Marinos, but was not found by Bonner and Foster or Hancock. In the present experiments it occurs in some but not all of the experiments. The rapid initial expansion is not an invariable part of the response of sections to high levels of auxin but is a phase which occurs under certain conditions.

Attempts have been made to determine the specific conditions which cause the appearance or absence of the rapid initial expansion. The evidence obtained is chiefly of a negative nature. The rapid initial expansion does not occur in the absence of active metabolism. There is no apparent relation between this process and the uptake of auxin into the tissue as a whole. Its appearance does not seem to be due to the use of tap water during the growing of the seedlings. Determination of the nature of this process must await further evidence.

ACKNOWLEDGEMENT

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Studies on the Geotropism of Roots

III. EFFECTS OF GEOTROPIC STIMULATION ON GROWTH-SUBSTANCE CONCENTRATIONS IN *VICIA FABA* ROOT TIPS*

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With three Figures in the Text

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SUMMARY

The auxins contained in 5-mm. tips of horizontal *Vicia faba* roots have been compared with those in tips of vertical roots after cold ethanol extraction, paper-chromatographic separation, and *Avena* mesocotyl bioassay. At about the time curvature commences in horizontal roots there is a marked increase in the content of an auxin corresponding to 'AP(*ii*)' of pea roots (Rf 0·35–0·65 in isobutanol/methanol/water). There are indications that this is not due to its release from an inactive bound state but that it is either synthesized *de novo* or may be converted from another auxin corresponding to 'AP(*iii*)' of pea roots (Rf 0·75–1·0).

The literature dealing with the auxins of geotropically stimulated organs is reassessed and it is concluded that, with the exception of the *Avena* coleoptile, there is very little evidence favouring a simple transport redistribution of auxin under gravity; the majority of the data favour an effect of gravity on auxin metabolism.

INTRODUCTION

FROM a previous investigation on the effects of externally applied auxins on the responses of roots of *Pisum sativum* to lateral geotropic stimulation it was concluded that the reduced overall growth-rate and the growth curvature induced could best be explained by a burst of growth inhibitor production in the lower half of the root tip (Audus and Brownbridge, 1957). It was further suggested that this could be the outcome of the activation, in the cells concerned, of enzymes which were either synthesizing the growth-controlling inhibitor or releasing it from an inactive bound state. A proper test of this hypothesis would involve the analysis of growth-substance changes in both the upper and lower halves of root tips during the course of the curvature responses to geotropic stimulation. Such technically difficult experiments are still in the development stage but some light has already been thrown on the problem by the measurement of changes in growth-substance concentrations in root tips as a whole. On the above theory one would expect

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increases in inhibitor content sufficient to account for a reduction in extension growth-rate of approximately 50 per cent. (see Audus and Brownbridge, 1957). The results of preliminary experiments of this kind are discussed in this paper.

METHODS

During the search for suitable techniques for slicing root tips into two precisely equal halves along the longitudinal axis it became obvious that the thicker roots of *Vicia faba* were much more easily handled than those of *Pisum sativum* used in the earlier work. A thorough analysis of the growth-substance content of seedling *Vicia faba* roots was therefore made, using solvent-partition and paper-chromatographic methods combined with coleoptile, mesocotyl, and root-segment assays, and these results have been reported elsewhere (Lahiri and Audus, 1960). The same methods have been used in this investigation, although, up to the present, attention has been confined to the acid ether-soluble fraction of cold ethanol extracts.

Three-day-old dark-grown *Vicia faba* seedlings were used with radicles approximately 6 cm. long. Since only 0·5 cm. of the tip was harvested for extraction, the accumulation of a sufficient quantity of material to allow reasonably precise estimates of the active materials in extracts (i.e. 1·0–1·5 gm.) necessitated the use of samples of 100–20 roots. Seedlings in samples of 15 to 20 were taken from the sterilized sand in which they had been grown, were washed in distilled water, and were then pinned through the cotyledons on to small cork mats covered with filter-paper. The mats were placed in glass tanks containing a small quantity of water so that the roots were vertical. To stimulate the roots individual mats were turned through 90° in a predetermined sequence and at appropriate intervals to allow for the time taken to harvest the tips at the end of the stimulation period. At harvest 0·5 cm. tips of the root were cut off and plunged into ethanol kept at 0° C. in a flask in an ice bath. The total fresh weight of each sample was determined by weighing the flask before and after addition of the tips. Similar samples of roots kept vertical were used as controls in all such stimulation experiments. During the experimental period the surfaces of the roots were kept moistened with distilled water by mist from an atomizer spray. The experiments were carried out at room temperature (18–20° C.) in diffuse low-intensity light (approx. 5–10 foot candles) away from the laboratory windows.

RESULTS

There are marked changes in the acid growth-substance content after stimulation, particularly in that auxin component corresponding to the substance AP(*ii*) of pea roots (Audus and Gunning, 1958). This is shown in Fig. 1 from an experiment in which roots were placed horizontal for 1 hour. It will be seen that the quantities of AP(*ii*), as measured by both the *Avena* mesocotyl-segment and the *Pisum* root-segment assay, are markedly increased in stimulated roots. Furthermore the mesocotyl assay indicates a fall in the

content of AP(*iii*) running at Rf 0·75–1·0, although this is not shown up in the pea-root assay.

To follow the kinetics of these changes, a series of experiments was carried out with stimulation periods of 15, 30, 35, 40, and 60 minutes respectively and estimates made of the concentrations of the three main auxins AP(*i*), AP(*ii*), and AP(*iii*) in each root sample.

In the previous survey (Lahiri and Audus, 1960) it has been demonstrated

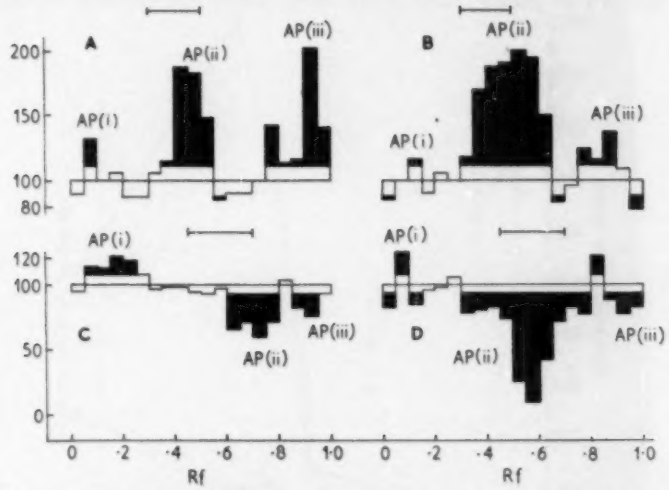

FIG. 1. Chromatogram showing the effect of geotropic stimulation on the growth-substance content of 5-mm. root tips of *Vicia faba* seedlings.

A and C—Extracts of vertical tips.
B and D—Extracts of tips of root placed horizontal for 1 hour.
A and B—*Avena* mesocotyl-segment assay.
C and D—*Pisum* root-segment assay.

The black portions of the histograms represent responses significant at the 5 per cent. level. The horizontal lines above the histograms mark the Rf positions of marker spots of indole-3-acetic acid.

that the concentration–activity curve of each of these substances differs markedly from that of IAA and so it was deemed undesirable to attempt to express these concentrations in terms of 'IAA equivalents' determined from a standard IAA calibration curve. Instead, an arbitrary measure of quantity has been obtained by simply determining the area of histograms corresponding to the three active spots and from these areas the corresponding 'concentrations' have been calculated by dividing by the fresh weights of the root samples. Only those portions of the chromatogram where the histogram rises above the fiducial limits of the assay have been included, but the areas have been calculated from the control line of 100. All histograms were comparable in that the growth of all assay samples was expressed as percentage

of control. Such estimates are, of course, subject to the variability introduced by day-to-day changes in the sensitivity of the mesocotyl material used in this series of experiments which will augment the already high variability arising in root sampling, extraction, and chromatography. But this can be

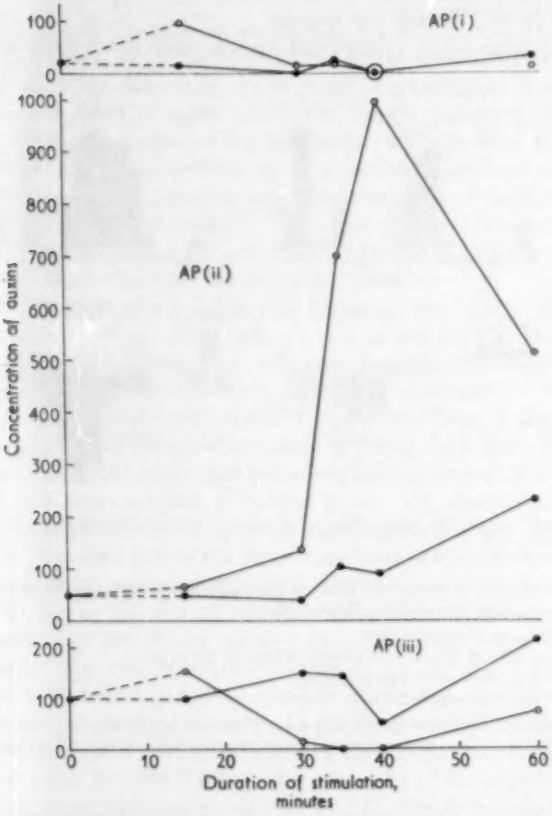

FIG. 2. Graphs showing changes in concentration of the three principal acid ether-soluble auxins in *Vicia faba* roots during the course of geotropical stimulation and the early stages of curvature.

Vertical roots —●— Horizontal roots —○—

avoided only when these substances have been identified and their true concentration–activity relations determined. The results of these experiments are brought together in Fig. 2.

In normal, vertically orientated roots the three auxins are present in approximately the following proportions:

$$AP(i):AP(ii):AP(iii)::20:50:100$$

During the first 20 minutes or so of stimulation there is little significant change in AP(*ii*) or AP(*iii*). After 30 minutes, however, the content of AP(*ii*) begins to rise rapidly and reaches an apparent peak at 40 minutes. Although there are considerable fluctuations in its content in vertical roots, it is clear that stimulation has resulted in a burst of production of this substance after 30–40 minutes of stimulation. After the same interval the content of AP(*iii*) in stimulated roots drops to vanishing proportions although there are no changes of this sort in vertical roots. With AP(*i*), which, in contrast to the

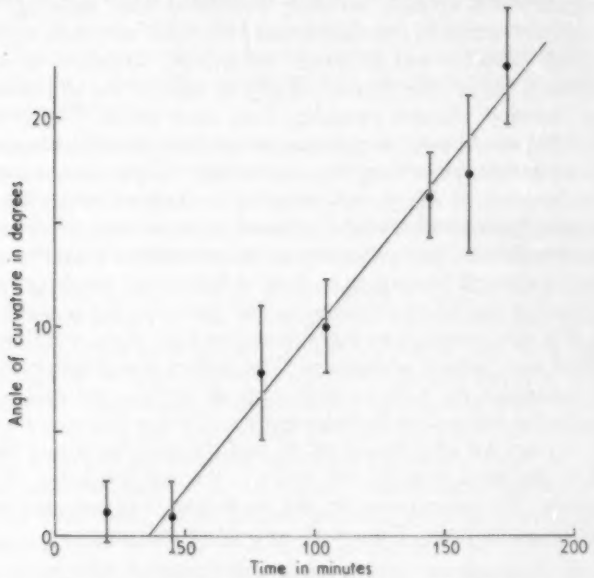

FIG. 3. Time-course of curvature in horizontal roots of *Vicia faba* seedlings. The points and vertical lines represent the mean curvature of a root sample ± the standard deviation respectively. The line through the points is the calculated regression line of curvature on time representing a rate of approximately 9° per hour. This cuts the time axis at a reaction time of 36 minutes.

other two substances, is a root-growth promoter (Lahiri and Audus, 1960), there are no consistent changes due to stimulation over the whole of this time range.

Observations have also been made on the onset of curvatures in roots under the same experimental conditions. For this, samples of six seedlings were placed in a slim glass-sided chamber and held in position by pads of moist cotton-wool. The long axes of the roots were all held parallel and out of contact with the glass walls of the chamber or the cotton-wool. The roots were geotropically stimulated in dim light but were illuminated for about one second with a 60-watt lamp at about 6 inches, when photographic records were taken at suitable intervals. The results of an observation of this kind appear in Fig. 3, where mean curvatures in degrees are plotted against time.

The initial rate of curvature is apparently constant and a regression line of curvature on time cuts the time axis at 36 minutes, which represents the best estimate of the reaction time. At this time, when curvature is just beginning, the concentration of AP(*ii*) is rising at about its most rapid rate.

These results would seem to imply that the reduced extension rate observed during geotropic curvature in roots (Audus and Brownbridge, 1957) could be due to this rapid accumulation of AP(*ii*), which is an active root-growth inhibitor. They also suggest that AP(*ii*) arises by enzymatic conversion from AP(*iii*), which shows changes inversely correlated with those of AP(*ii*). If this is so and the change is a straightforward chemical one, then, since AP(*iii*) is also a root inhibitor and an auxin, its activity, molecule for molecule, would be much lower than that of AP(*ii*), in view of the difference in the respective 'activity' changes resulting from stimulation. The root-growth promoter AP(*i*) would seem to play no part in these growth changes.

It is possible that AP(*iii*) may be a relatively simple compound formed by the combination of AP(*ii*) with some other inactive metabolite of small molecular size, from which AP(*ii*) is released by an enzyme generated during geotropic stimulation. This raises the question whether a similar release of AP(*ii*) from a state of binding on to large colloidal cell constituents such as proteins may not also be contributing to this dramatic rise in concentration. To check this, one preliminary experiment has been done to follow changes in both 'free' and 'bound' acid auxins. The former was determined as heretofore by extraction for 3 hours with ether at +2° C., the acid fraction of the extract being analysed on chromatograms. The root tips were then further extracted in ether for 48 hours at 28° C. and the acid fraction of the extract subjected to the same analysis, the auxin in this fraction being taken to be 'bound' auxin. In this experiment the stimulated root samples were kept horizontal for 40 minutes. The results are shown in Table I.

TABLE I

'Concentrations' of 'free' and 'bound' acid auxins in vertical and horizontal root tips of Vicia faba

		AP(*ii*)	AP(*iii*)
Vertical	Free	61	281
	Bound	48	153
Horizontal	Free	201	369
	Bound	70	231

Unaccountably, in this experiment, stimulation seems to have increased the content of AP(*iii*) as well as AP(*ii*) although it must be admitted that this rise in AP(*iii*) is of the order of the variability noted in the previous work. This occurred in both 'free' and 'bound' fractions, the ratio remaining virtually the same in both control and stimulated roots. 'Free' AP(*ii*), however, showed more than a threefold rise, which is below that seen in the previous experimental series for the same stimulation time but is certainly well above the limits of error. The 'bound' fraction also shows a rise which is much

smaller and probably not significant. These results, therefore, give no support for the suggestion that the upsurge of AP(*ii*) is caused by its release from an inactive bound form and point to a *de novo* synthesis from an inactive precursor. The smaller but parallel changes in AP(*iii*), which conflict with the results of the kinetic experiments, underline the need for further work along these promising lines.

DISCUSSION

This convincing demonstration of a burst of auxin *production* in geotropically stimulated roots, which runs counter to the classical and still generally accepted theory of response mechanism based on auxin *redistribution*, calls for a reappraisal of the earlier evidence. The classical theory is based on the two following claims:

(*a*) That there is no geo-growth reaction and hence, by implication, no change in the overall concentration of growth-regulating substances in the elongating and curving zone of the organ.

(*b*) That direct assay shows no change in total auxin concentrations, the upper side of a stimulated organ losing as much auxin as the lower side gains.

The pioneering work on which this is based is that of Dolk (1936), who studied the phenomena in the coleoptiles of *Avena* and *Zea*. Rotation of coleoptiles with their axes parallel to the horizontal axis of a continuous klinostat caused no change in growth-rate, indicating no geo-growth reaction. But to avoid the complication of nutations the tips were laterally restrained by a fine ring. In view of the modification of geotropic response in roots by the mechanical effects of the surrounding media (Bennet-Clark *et al.*, 1959) this procedure must be regarded as not entirely satisfactory. Similar results not open to the same objection have been obtained by Brain (1935), while Bara (1957) has demonstrated a growth acceleration under the same conditions. In *Avena* coleoptiles freely curving under gravity no change in growth-rate was observed (Navez, 1933; Navez and Robinson, 1933). Although the situation is still not clear-cut it would seem that, in *Avena* coleoptiles at least, the available evidence supports contention (*a*) above.

But in other organs, particularly in roots, there is overwhelming evidence for strong geo-growth reactions. Thus slow rotation on a horizontal axis increases the growth-rate of *Helianthus* hypocotyl, *Asplenium bulbiferum* fronds, *Narcissus* leaves, and peduncles and coleoptiles of *Zea* (Brain, 1935; Bara, 1957). In most species tested the growth of roots is depressed by placing them horizontal. This has been established under rotation on a klinostat for *Lens culinaris* (Bara, 1957), *Artemisia absinthium* (Larsen, 1953), *Avena sativa*, *Lupinus albus*, and *Helianthus annuus* (Brain, 1935), and during normal curvature after stimulus in *Sinapis* and *Vicia faba* (Bennet-Clark *et al.*, 1959) and *Pisum sativum* (Audus and Brownbridge, 1957). An exception seems to be *Zea mays*, where growth is accelerated both when roots are curving normally (Keeble, Nelson, and Snow, 1931) and when they are rotating on the horizontal klinostat (Brain, 1935).

All this suggests that the coleoptile of *Avena* is an exception to the rule, and it is therefore unfortunate that its behaviour has formed the basis of a general theory of geotropic response. Certainly the remaining data for all other organs point to a marked disturbance of overall growth-rates which is most likely to be the reflection of correlated changes in one or more growth-regulating substances.

This situation, then, gives us an incentive to look again at the classical data for evidence of changes in total content of growth substances during geotropic response. Taking again the pioneering work of Dolk (1936), direct comparison of the diffusible auxin from tips of vertical and horizontal coleoptiles of *Avena* and *Zea* shows that there is no significant difference to be detected, which is in agreement with the absence of a geo-growth response. However, if data from the auxin-distribution experiments are compared with those from normal tips some differences appear. Dolk's own comparisons are made between auxin contents of samples with a varying number of tips. If his results are recalculated on a *per tip* basis then the following results emerge:

Tips from whole vertical coleoptiles . .	$1{\cdot}07 \pm 1{\cdot}1$	auxin 'units'	per tip
Tips from whole horizontal coleoptiles . .	$1{\cdot}09 \pm {\cdot}09$	,, ,,	per tip
Upper half-tips from horizontal coleoptiles .	$0{\cdot}68 \pm {\cdot}18$	,, ,,	per half-tip
Lower half-tips from horizontal coleoptiles .	$1{\cdot}07 \pm {\cdot}14$	,, ,,	per half-tip
Sum of auxin from both halves . . .	$1{\cdot}75 \pm {\cdot}31$	,, ,,	per tip

A 't' test shows the difference (i.e. 0·67) between this last sum and the content of vertical tips to be on the 5 per cent. significance level. Dolk concluded that the auxin content of horizontal coleoptiles as calculated by summing the figures from upper and lower halves did not differ within the limits of assay variability from the content of vertical coleoptiles. The significant increases shown by this analysis, which might lead one to suspect an effect of geotropic stimulation, indicate some experimental inconsistencies and point to the need for further, more standardized, observations. Fortunately, however, other independent observations support Dolk's contentions (Navez and Robinson, 1933; Gillespie and Briggs, 1959); unfortunately his conclusions have been applied indiscriminately and without direct experimental check to other organs. Presumably on the assumption that lateral transport of auxin under gravity was the only phenomenon that would operate, most workers have measured auxin *distribution* in horizontal organs without taking the trouble to compare the concentrations with those in vertical organs. This is so for experiments on roots of *Vicia faba* (Hawker, 1932; Amlong, 1937; Boysen Jensen, 1933, 1936) and for epicotyls of *Vicia faba* (van der Laan, 1934; Boysen Jensen, 1936). The claims from the meagre and variable results of Dijkman (1933, 1934) that there are no overall auxin changes induced in hypocotyls of *Lupinus albus* are made doubtful by the more convincing results of Brain (1942), whose data show a considerable increase in auxin content in horizontal hypocotyls on a klinostat.

There is no doubt that the stimulus of gravity can result in a marked

change in auxin metabolism in some organs. As early as 1933 Schmitz showed that in the mature nodes of a number of grasses auxin is rapidly generated when the haulm is laid horizontal. These results seem quite unambiguous since normal vertical nodes contained no auxin detectable by his assay. On the whole more auxin was produced on the under sides of these nodes. Later, van Overbeek *et al.* (1945) showed the same phenomenon in the nodes of sugar-cane, where the greatest increases took place in the growth rings at either end of the node. But in comparison with the results of Schmitz, which were for diffusible auxin, the ether-extracted auxin here showed no differences in concentration between upper and lower sides, which pointed to a geotropic response mechanism differing from that in seedlings. The authors suggested that the differential growth was being mediated not by auxin but by the unequal distribution of another growth-limiting factor.

From all these observations it will be clear that, with the possible exception of the *Avena* coleoptile, there is virtually no direct evidence that auxin concentration differences in horizontal organs are brought about purely by lateral redistribution of its normal auxin content. Even in *Avena* direct observation following applications of ^{14}C-labelled indole-3-acetic acid could establish no lateral transport of this kind (Te May Ching and Fang, 1958). Furthermore, it must not be forgotten that all the foregoing observations are based on the assay of crude diffusates or extracts possibly containing a complex of growth promoters and inhibitors whose integrated growth effects may give no clue to the relative contributions of the separate components. For example, results indicating decreases or increases in promoter content may equally well be due to inverse changes in inhibitors or more likely to a combination of both.

This survey of the literature, taken together with the experimental results described in this paper, in our opinion strongly favours a metabolic basis for the observed growth-substance distribution in geotropically stimulated organs. Such metabolic changes may well be complex, involving more than one active growth-regulating substance. There is therefore urgent need for a new and completely open-minded approach to this problem, using the relatively precise technique of chromatography as an essential preliminary to biological assay and chemical identification, in which the kinetics of the changes in all growth-active materials in the upper and lower parts of curving organs are carefully followed. Only then shall we be able to draw any conclusions as to the compounds responsible for the curvatures and the mechanisms involved in the establishment of the necessary concentration differences. As far as we are aware no such investigations have yet been published although work is under way in more than one laboratory.

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Evidence for a Protein Gel Structure Cross-linked by Metal Cations in the Intercellular Cement of Plant Tissue[1]

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With eight Figures in the Text

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SUMMARY

The factors which modify the action of chelating agents in dissolving the intercellular cement in plant tissue have been investigated. The action was assessed by determining the degree of separation of cells in treated pea root tips.

Greatly increased separation was observed when heavy metal chelating agents were combined with EDTA (which, in addition to heavy metals, chelates Ca) indicating that two types of cations are involved in stabilizing the cement. After the cells had been separated by removal of the cations they could be recemented by treatment with divalent cations. In low concentrations only certain combinations of cations were effective, again pointing to the presence of more than one type of cation in the cement. Proteolytic enzymes or denaturing agents eliminated this capacity to recement. Pretreatment with divalent cations enhanced subsequent separation (in EDTA solution). This effect could be countered by treatment with monovalent cations.

Pretreatment in buffers covering a wide range of pH resulted in increased separation both at pH 3 and at pH 11, suggesting an ampholytic component in the cement.

Urea, when applied as a pretreatment, only enhanced separation when in high concentration. This effect was influenced by ionic strength. When applied in combination with EDTA, urea produced an optimum effect at low concentration (0.125 M.). Pretreatment in hot water also enhanced subsequent separation in EDTA solutions. High concentrations of KCl countered this effect. These findings point to the importance of H bonds in the intercellular cement. Thioglycollic acid, applied either as pretreatment or simultaneously with EDTA, lowered the EDTA concentration necessary to achieve separation, suggesting the presence of S—S bridges.

The curve relating the effect of pretreatment at varying temperatures, either in H_2O or in apolar solvents, to temperature, showed a sharp break, suggesting that the melting-point of an organized gel might be involved.

The conclusion is reached that the intercellular cement can be regarded as an oriented gel structure containing protein molecules cross-linked by two types of metallic ion, the metallic cross linkage being chelate in character.

INTRODUCTION

In a previous paper (Ginzburg, 1958) it was suggested that the middle lamella of plant tissue contains a protein component in addition to the known pectic substances. This suggestion was based on the action of chelating agents in

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effecting the separation of the cells in tissue. Experiments with ethylene-diamine tetra-acetic acid (EDTA) showed that the reaction proceeded only in the presence of rather high concentrations of the chelating agent; the rate of the reaction was rather slow and its Q_{10} was very high. The following working hypothesis was postulated. The middle lamella is stabilized by a metal ion; this metal is hidden inside the structure so that it is not easily accessible to the chelating agents. It seemed reasonable to assume that such a structure is a part of a macromolecule or an aggregate of macromolecules. It has further been shown that various denaturing agents enhanced the separating activity of the chelating agents; this is compatible with the idea that protein is a component of the structure.

The present paper describes experiments designed to test the above working hypothesis and to clarify the nature of the structure which binds plant cells together, i.e. the 'intercellular cement'. Experiments were designed in which the action of the chelating compounds was modified by various agents which are thought either to destroy or to weaken the structure hiding the metal ion, thus making the metal ion readily available to chelation and resulting in a rapid dissolution of the intercellular cement.

METHODS

The material used was 1 mm. root tips taken from Alaska pea seedlings which had been grown for 45–50 hours in Petri-dishes in the dark at 25° C. The root tips were immersed in 1–2 ml. of the test solution in small vials, and at the appropriate times placed on slides in a drop of distilled water or the solution to be tested. By tapping gently on the cover-glass, separation of cells in the root tips was brought about. Under the microscope the percentage separation could be estimated. The quantity estimated corresponded approximately to the percentage of single (completely separated) cells, but the degree of separation into blocks of cells, and the size of these blocks, also influenced the estimate. The degree of precision of the estimate is indicated by the fact that variation between several replications was almost invariably less than 10 per cent. Every treatment was carried out in duplicate, and most of the experiments were repeated three times. The values given in the tables and figures are the means of several experiments. Double-distilled water from a glass still was used throughout the experiments. Except where otherwise indicated the ethylene diamine tetra-acetic acid (EDTA) solutions were brought to pH 8·0 with NaOH.

RESULTS

1. *Ionic phenomena*

Effect of ionic strength. The curve relating separation of cells to EDTA concentration is not asymptotic, as might have been expected, but shows an optimum. The polyanionic nature of EDTA at the pH values used suggested that the decline of the curve at post-optimal EDTA concentrations might be related to the ionic strength of the solution: where ionic strength is high, even

though intercellular cement has been weakened, separation of cells may be prevented by electrical forces between the cells (cf. Mazia, 1954). Two types of experiment were designed to test this assumption. First, root tips placed in a series of EDTA concentrations for certain lengths of time were removed and either examined at once or examined after rinsing for 10 minutes in distilled water at room temperature. As will be seen from Fig. 1, rinsing prevented the usual decrease in percentage separation at supraoptimal EDTA

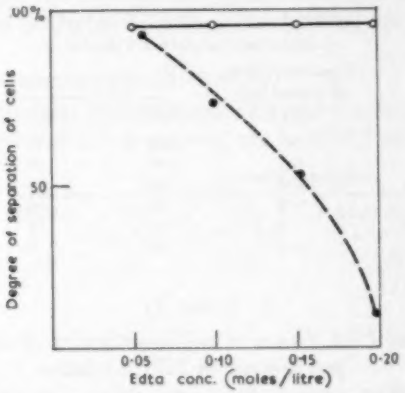


FIG. 1. The effect of post-treatment with distilled water on the separation of cells in pea root tips treated with high concentrations of EDTA. (pH 8·0, temperature 25° C., experimental period 32 hours.)
●——● examined immediately after EDTA treatment.
○——○ examined after 10 minutes in distilled water.

concentrations. In the second type of experiment the ionic strength was altered by addition of other ions to the optimal concentration of EDTA. Table I shows the effect of various concentrations of KCl and LiCl on the separation obtained in 0·1 M. EDTA. Increasing salt concentration decreases separation. It seems reasonable to assume from the two types of experiment that high ionic strength is the cause of the decrease in percentage separation at high EDTA concentration. This points to the existence of a charge either in the intercellular cement or on the cell surface.

Evidence for presence of metal ions in the intercellular cement. Heath and Clark (1956 *a*, *b*) have shown that while low concentrations of certain chelates do not affect growth of wheat-coleoptile sections, combinations of low concentrations show marked activity. It was unimportant which combinations were used. Combination of a wide range of EDTA concentrations with various concentrations of diethyldithiocarbamate (Dieca), *o*-phenanthroline, and indoleacetic acid (IAA) were accordingly tested. It was found that

separation was obtained only when the EDTA concentration was at least 0·1 M. It will be seen in Table II that even very low concentrations of the chelates markedly promoted the action of 0·1 M. EDTA. Various combinations of chelates other than EDTA were also tested, in the concentration range of

TABLE I

The effect of added KCl and LiCl on the separation of cells in pea root tips treated with EDTA

(*EDTA 0·1 M., pH 8·0, temp. 37° C., experimental period 8 hours*)

Concentration of added salt (M.)	% cells separated	
	KCl	LiCl
0	100	100
0·1	100	90
0·6	22	30
1·0	22	25
1·2	15	15
1·5	10	8

TABLE II

The effect of added IAA, Dieca, or o-Phenanthroline on separation of cells in pea root tips in EDTA solution

(*EDTA 0·1 M., pH 8·0, temp. 25° C., experimental period 14 hours*)

Concentration of additive (M.)	% cells separated		
	IAA	Dieca	*o*-Phenanthroline
0	20	25	25
10^{-7}	60	95	55
10^{-6}	52	65	55
10^{-5}	52	92	55
10^{-4}	45	100	85
10^{-3}	50	100	85

10^{-2} M.–10^{-7} M. Negative results were obtained in all cases except 10^{-2} IAA with either 10^{-3} M. Dieca or 5×10^{-3} M. *o*-phenanthroline (20 per cent. separation); and 10^{-3} M. Dieca with 5×10^{-3} M. *o*-phenanthroline (20 per cent. separation). Where positive results were obtained the characteristic red colour of the Fe-*o*-phenanthroline complex, or the brown colour of the Cu-dieca complex, were observed.

It was next decided to try and separate the action of EDTA from that of the other chelates. The root tips were pretreated with the chelates and then with EDTA and vice-versa (Table III). In both cases the separation of the two treatments resulted in increased separation of the cells. However, the chelate concentrations required are higher than those effective when they are applied simultaneously with the EDTA.

These findings, i.e. (*a*) that, in contrast to the results of Heath and Clark (1956*a*, *b*), the choice of the chelate combinations was extremely important;

and (*b*) that good separation was effected only in the presence of high EDTA concentration, suggest that two actions are involved in the separation of the cells. *o*-phenanthroline, Dieca, and IAA are all heavy metal chelates. Their actions may be ascribed to their chelation of Cu and Fe. This conclusion is supported by the appearance of the coloured complexes when Dieca and *o*-phenanthroline cause separation, and their failure to appear in the absence of separation. EDTA, however, chelates not only heavy metals but also forms a strong chelate with Ca and Mg. Its special effect on separation may be ascribed to the latter property which the other chelating agents do not possess.

TABLE III

The effect of pretreatment or post-treatment with heavy-metal chelating agents on separation of cells in pea root tips in EDTA solution

	% cells separated				
Treatment	Water	Dieca 10^{-2} M.	IAA 10^{-2} M.	*o*-phenanthroline $5 \cdot 10^{-3}$ M.	EDTA 10^{-1} M.
21 hrs. in specified chelating agents followed by 14 hrs. in EDTA	10	45	45	40	—
15 hrs. in EDTA followed by 21 hrs. in specified chelating agents	20	37	40	30	55

The following interpretation of these results is possible. The intercellular structure consists of a macro-molecular complex stabilized by two types of metal ion. The removal of one of these two types of ion facilitates the removal of the second ionic type. This results in the weakening of the intercellular cement and the separation of the cells. When applied simultaneously with EDTA very low concentrations of heavy metal chelates are effective. This might possibly be explained if, owing to their greater mobility (resulting from their smaller size and, especially, their lower charge), the heavy-metal chelating agents are able to act as carriers of the metal ions between their position in the macro-molecular framework and the EDTA (see Discussion).

Effect of pretreatment with salts on separation. In a previous paper (Ginzburg, 1958) it was suggested that the action of auxin in growth is due to its effect on the metal ions present in the intercellular cement. Cooil and Bonner (1957) have shown that Ca, Mg, Sr inhibit the effect of IAA on growth. This inhibition can be reversed by K salts or by leaching of the tissue by distilled water. This suggests that the inhibiting ions are bound in exchangeable form. It was therefore considered that it might be possible to replace Ca or other ions in the intercellular cement by monovalent ions. This might be achieved

TABLE IV

The influence of pretreatment with 3 concentrations of various salts on the separation of pea root tips treated with EDTA

(EDTA 0·1 M., pH 8·0; temp. 25° C., experimental period 14 hours)

Pretreatment	% separation of cells		
	1·0 M.	10^{-2} M.	10^{-4} M.
Control (water)	22	22	22
KCl	22	22	30
LiCl	35	40	40
$CaCl_2$	—	60	90
$CoCl_2$	65	80	55
$MnSO_4$	90	80	65
$ZnSO_4$	32	70	90
$MgSO_4$	75	70	70

TABLE V

The influence of pretreatment with salts for varying periods on the separation of cells in pea root tips subsequently treated with EDTA

(EDTA 0·1 M., pH 8·0, temp. 25° C., experimental period 16 hours)

Pretreatment		% cells separated					
		Pretreatment at 25° C. *Pretreatment period*			Pretreatment at 2° C *Pretreatment period*		
		15 min.	3 hrs.	25 hrs.	15 min.	3 hrs.	25 hrs.
Control (water)		20	30	30	22	17	22
$Fe(NO_3)_3$	10^{-2}	60	95	100	27	80	75
	10^{-4}	10	50	55	38	45	35
	10^{-6}	20	45	—	27	45	25
$Cu(NO_3)_2$	10^{-2}	65	65	65	22	65	55
	10^{-4}	45	77	37	27	40	37
	10^{-6}	32	70	65	27	55	37
$Ca(NO_3)_2$	10^{-2}	22	55	52	22	37	55
	10^{-4}	40	55	50	20	37	55
	10^{-6}	35	70	35	20	50	30
$BaCl_2$	10^{-2}	0	0	0	0	0	0
	10^{-4}	0	0	0	0	0	0
	10^{-6}	20	30	8	8	8	32
MnCl	10^{-2}	55	85	32	55	55	60
	10^{-4}	40	70	85	35	60	55
	10^{-6}	32	75	80	40	55	95

by treating the tissue with high concentrations of various ions. Divalent ions were also tried, as Heilbrunn (1956) showed that the changing of Ca proteinate to Mg proteinate resulted in conversion of a gel to a sol.

Root tips were immersed in solutions of KCl, LiCl, $MgSO_4$, $CoCl_2$, $ZnSO_4$, $CaCl_2$, and $MnSO_4$ in concentrations from 0·001 M.–1·0 M. for periods of from 15 minutes to 18 hours. In no case did treatment with the salts achieve separation. Apparently the monovalent ions do not exchange with the ions present in the cement, since, had exchange occurred, separation would have been expected. Exchange of divalent ions would not necessarily have led

to separation, but it is possible that the resultant structure would be more readily attacked by EDTA. Tissue treated with salts was placed in EDTA and degree of separation compared with that in non-treated controls. Results are given in Table IV. It will be seen that pretreatment with KCl was ineffective, LiCl had some effect which was independent of concentration, and all the divalent cations very appreciably affected the separation. The influence of length of pretreatment, temperature, and concentration is shown in Table V. It will be seen that even 15 minutes' pretreatment had a marked effect. No appreciable effect of temperature can be observed. In some cases even very

TABLE VI

The counteraction by KCl of the influence of pretreatment with $Ca(NO_3)_2$ or $Cu(NO_3)_2$ on the separation of cells in pea root tips

(The root tips were first placed in 10^{-2} M. nitrate solutions for 1 hour. They were then transferred to KCl for 3 hours, after which they were placed in 0·1 M. EDTA, pH 8·0, for 14 hours at 25° C.)

Concentration of KCl M.	% separation of cells		
	H_2O	$Ca(NO_3)_2$	$Cu(NO_3)_2$
0	15	60	75
0·01	10	45	65
0·1	12	40	50
1·0	8	25	5
3·0	15	25	10

low concentrations, 10^{-6} M., were very effective. It will also be noted in Table V that Ba not only did not promote separation, but even inhibited the separation achieved by EDTA. Similar results, not recorded here, were obtained with Sr salts.

The rate of reaction and the low dependence on temperature suggest an ionic exchange reaction. However, the relatively slight dependence on concentration of the salt during pretreatment is not readily explicable, unless the affinity of the site for the entering ion is very much greater than that for the ion which is exchanged. Further, the fact that the native divalent ions cannot be replaced by potassium, while the divalent ions entering after pretreatment can, indicates that the site at which the native ions are held differs from that at which the ions entering after pretreatment are adsorbed. Monovalent ions are not effective during pretreatment, indicating that the second site is occupied by monovalent ions. The fact that K is quite ineffective while Li has a slight effect (Table IV) suggests that the normal monovalent ion present at the second site is potassium.

This conclusion is borne out by the following experiments involving a double pretreatment. The roots were first placed in a solution of divalent ions, transferred to KCl at various concentrations and then to EDTA as in the previous experiments. As will be seen from Table VI, high concentrations of KCl (1·0 or 3·0 M.) completely abolished the effect of pretreatment with a divalent ion. Even 0·1 M. KCl was to some extent effective. This experiment

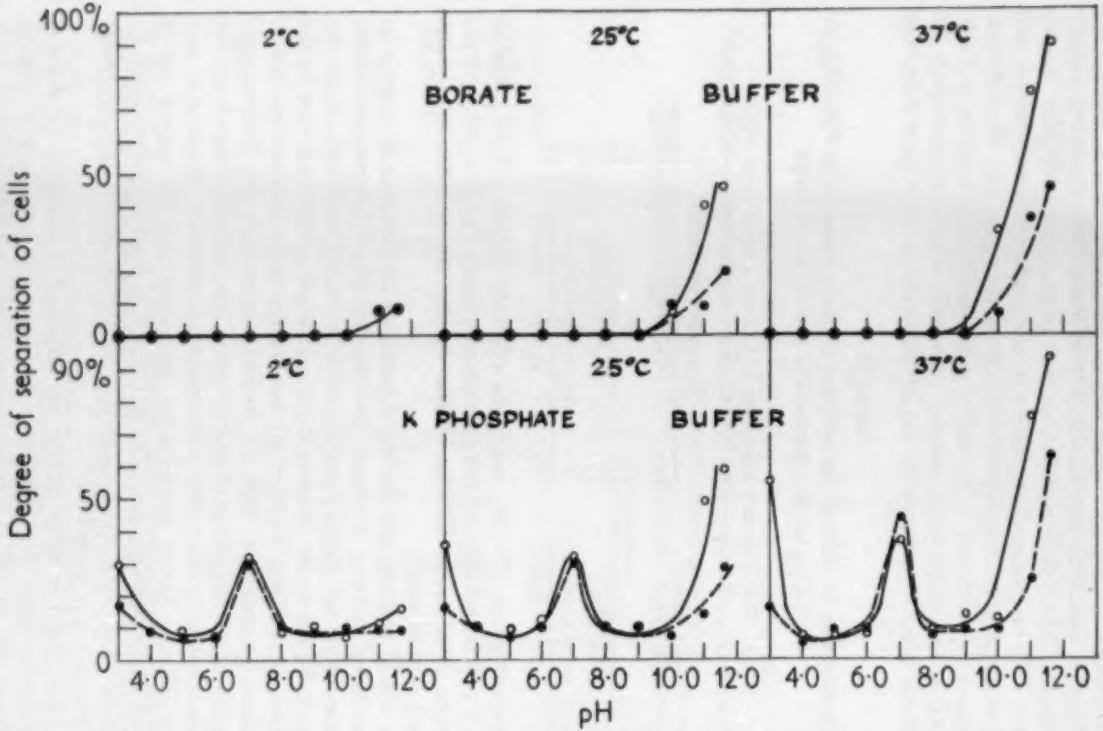


FIG. 2. The influence of pretreatment in buffers of varying pH on the subsequent separation of cells in pea root tips in EDTA solution. (EDTA 0·1 M., pH 8·0, temperature 25° C., experimental period 15 hours. Buffers 0·067 M.)

●– – –● 2 hours' pretreatment at indicated temperature. ○——○ 24 hours' pretreatment at indicated temperature.

thus confirms that K may be the ion native to the second site. This is in fact likely since it is abundantly present in plant tissue.

The picture which emerges from the experiments on pretreatment is thus as follows: Two sites are involved in these experiments. Monovalent ions, probably potassium, are present at one site. Replacement of these ions by divalent ions alters the structure in such a way that the ions at the other site, which stabilize the intercellular cement, become more susceptible to attack by EDTA.

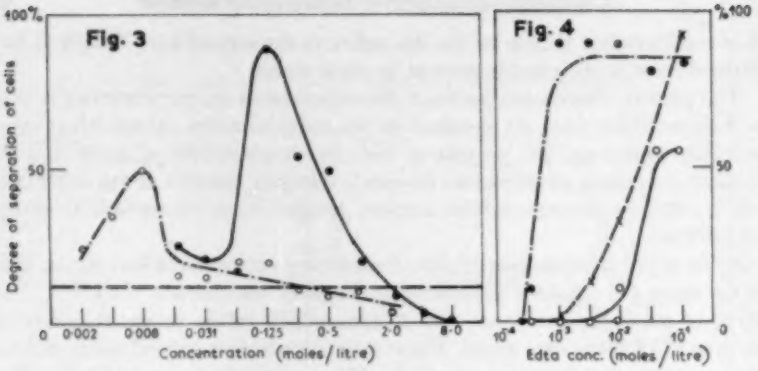
Effect of pH on separation of cells. Preliminary experiments had shown that in the range pH 2·2–12·0 no separation occurs in the absence of EDTA. The effect of pretreating the root tips at various pH values before transferring them to EDTA was next tested. These experiments were carried out at various temperatures, and for various periods. The results are shown in Fig. 2. The most striking result is that relatively high percentage separation was observed not only at the acid and alkaline extremes of the range tested, but also at about pH 7. This peak at about pH 7 was observed for all experimental periods and for all buffers tested (phosphate, tris, veronal) with the exception of borate. The effect of pretreatment at pH 7 was independent of the temperature and almost instantaneous. This indicates a reaction with a low energy of activation and suggests an electrostatic one. The peak of pH 7 may be at the isoelectric point of the protein of the intercellular cement. At the extreme pH values the effect of pretreatment is even more marked and shows some temperature and time dependence. This is in accord with the supposition that a protein component is involved. At extreme pH values the protein will be highly charged, causing structural changes resulting in easier access for EDTA.

The behaviour in borate buffers differs markedly from that in the other buffers. No separation resulted from pretreatment in borate buffer in the range of pH 3–11. Even the slight degree of separation obtained without pretreatment was absent. The borate ion appears to have a very specific effect on the site attacked by EDTA. This may be due to complexing with vicinal OH groups present near the site of attack as a result of which access to the latter becomes completely blocked. Such complexing may be with pectins since borate is well known to react with OH groups in various carbohydrates.

2. *Hydrogen bonds as stabilizers of the intercellular cement*

In previous experiments (Ginzburg 1958) it was shown that urea markedly enhanced the effectiveness of EDTA in separating the cells. Urea is generally regarded as an agent breaking H bonds (Kautzman, 1954, 1956; Mirsky and Pauling, 1936). It was therefore suggested that H bonds were involved in the structure of the intercellular cement. To test this suggestion the effect of urea has been examined in greater detail. In addition, other agents known to break H bonds, such as guanidine-HCl (Kautzman, loc. cit.) and hot water, have been tested. The effect of thioglycollic acid, which breaks both H and S—S bonds, has also been studied.

The effects of urea, guanidine hydrochloride and thioglycollic acid. Fig. 3 shows



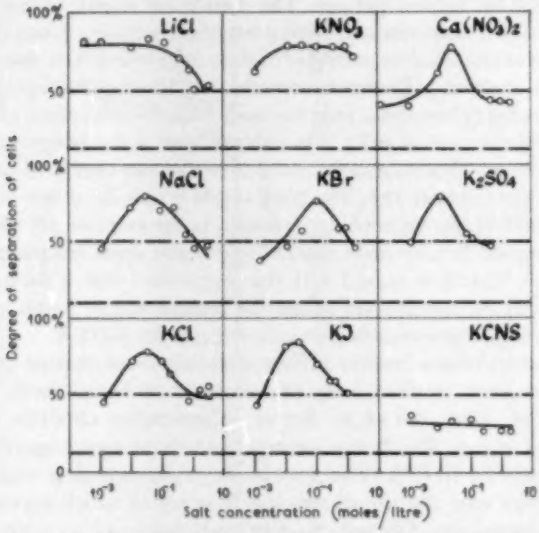


Fig. 5

FIG. 3 The effect of concentration of urea or guanidine-hydrochloride on the separation of cells in pea root tips treated with EDTA. (EDTA 0·1 M., pH 8·0, temperature 25° C., experimental period 14 hours.)

— — — — EDTA alone.
○—·—·○ EDTA + guanidine-hydrochloride.
●——● EDTA + urea.

FIG. 4. The effect of added urea (0·125 M.) or thioglycollic acid (1 per cent., pH 8·0) on the relation between separation of cells in pea root tips and EDTA concentration. (pH 8·0, temp. 25° C., experimental period 24 hours.)

○——○ EDTA.
×— — —× EDTA + urea.
●—·—·—● EDTA + thioglycollic acid.

FIG. 5. The effect of various salts when added to urea during pretreatment on the subsequent separation of cells in pea root tips in EDTA solution. (EDTA 0·1 M., pH 8·0, temp. 25° C., experimental period 14 hours.)

— — — — Pretreatment with water.
—·—·—· Pretreatment with urea 8·0 M.
○——○ Pretreatment with urea + salt.

the curve relating the effect of both urea and guanidine hydrochloride, when these are applied together with EDTA, to the concentration of these substances. The optimum for guanidine is at a much lower concentration, 0·008 M., than that for urea, 0·125 M. The maximum stimulation achieved by urea is, however, higher, showing it to be more effective than guanidine hydrochloride. These results are remarkable for the fact that low urea concentrations are effective. Generally the denaturing action of urea is associated with very high concentrations (Kautzman, 1954, 1956; Lindstrom-Lang, 1952; Putnam 1953). Kautzman, in interpreting this concentration requirement, suggests that the apparent high order of reaction, 12–14, in the case of ovalbumin is associated with the simultaneous breaking of a large number of H bonds.

TABLE VII

The effect of pretreatment with urea, guanidine-hydrochloride, or thioglycollic acid on the separation of cells in pea root tips in EDTA solutions

(EDTA 0·1 M., pH 8·0, temperature 25° C., experimental period 14 hours)

	% cells separated					
	2° C.		25° C.		37° C.	
Pretreatment	10 min. pretreatment	23 hrs. pretreatment	4 min. pretreatment	23 hrs. pretreatment	5 min. pretreatment	23 hrs. pretreatment
Water	15	15	15	15	10	15
Urea 0·125 M.	10	12	10	10	15	32
2 M.	15	12	17	25	27	12
6 M.	27	25	30	35	30	12
8 M.	45	35	50	45	10	10
Guanidine						
0·0125 M.	10	17	10	15	10	35
0·125 M.	15	35	20	20	12	30
4 M.	55	35	45	37	10	17
Thioglycollic acid 1%						
pH 8·0	22	30	15	35	22	75
pH 10·0	20	15	12	40	15	100

The effect was studied further at various temperatures, as it is known that the action of urea as a denaturing agent sometimes shows a negative temperature coefficient (Hopkins, 1930; Lindstrom-Lang, 1952, Kautzman, 1954, 1956; Johnson *et al.*, 1954). It was found that the effect of temperature on the action of EDTA and urea or guanidine hydrochloride is similar to that on EDTA alone (see Ginzburg, 1958), in the range of 2–40° C. No negative temperature coefficient was observed.

The effect of urea or thioglycollic acid on the EDTA concentration required to achieve 50 per cent. separation is shown in Fig. 4; the EDTA concentration required was almost the same in the presence and absence of urea. However, thioglycollic acid appreciably reduced the EDTA concentration necessary.

The effect of pretreatment of the root tips was next examined. Experiments

were carried out in which concentration, temperature, and time of pretreatment were varied. The results are given in Table VII, where it will be seen that urea is effective only at high concentrations, 6·0–8·0 M. The effect is very rapid, reaching a maximum after about 10 minutes. The temperature dependence is somewhat peculiar. There was no difference between the effect of urea at 2° C. and 25° C., but at 37° C. the effect was abolished. Guanidine hydrochloride behaved in an exactly similar manner. The effect of pretreatment by urea and guanidine hydrochloride was reversed by immersion of the root tips in water for 1 hour before applying the EDTA

TABLE VIII

The effect of a post-treatment in water on the separation of cells in root tips pretreated with urea, thioglycollic acid, or hot water

(*The root tips were first placed in pretreatment solutions for the specified periods, then transferred to water at 20° C. and subsequently to 0·1 M. EDTA, pH 8·0, temp. 25° C. for 15 hours*)

	% cells separated		
	Period in cold water		
Pretreatment	0 min.	5 min.	60 min.
H_2O	15	15	15
Urea 8 M. for 23 hours 2°	35	—	12
Urea 8 M. for 23 hours 25°	45	—	15
Urea 8 M. for 23 hours 37°	10	—	37
Thioglycollic acid 1% pH 8·0 for 23 hours at 37° C.	75	—	75
Thioglycollic acid 1% pH 10·0 for 23 hours at 37° C.	100	—	85
H_2O 90° C. 5 min.	55	40	10
H_2O 90° C. 15 min.	55	20	5

(Table VIII). Thus, in contrast to the results obtained when urea and EDTA were applied simultaneously, urea when applied as a pretreatment acted in accordance with its known behaviour as a denaturing agent, as described by Kautzman for ovalbumin, and Lindstrom-Lang for lactoglobulin.

Thioglycollic acid affected separation in an entirely different manner. The effect was slow and temperature-dependent, the Q_{10} being about 2 in the range 25°–37° C. (Table VII); the effect was not reversible. Thioglycollic acid is known as a feeble H bond breaker (Lloyd and Garrod, 1948). It does, however, readily and specifically break and reduce S—S bonds (Barnett and Seligman, 1954). It therefore seems probable that the action of this acid was due to its effect on S—S bonds present in the intercellular cement.

Kautzman (1954) has shown that the denaturing action of urea can be modified by various ions. Doty (1957) suggests that high ionic strength inhibits H-bond breaking by urea and heat in the case of denaturation of DNA. The effect of ionic strength, and of the nature of the ion, on the effectiveness of urea pretreatment was therefore investigated. The results are shown in Fig. 5. It will be seen that NaCl, KCl, KI, KBr, $Ca(NO_3)_2$ and K_2SO_4 all show a

similar effect when applied to the root tips together with 8·0 M. urea during pretreatment. All of these produce their optimum effect at similar concentration. This suggests that ionic strength is involved. The anions Cl^-, Br^-, and I^- all behave similarly.

One cation, Li^+, and two anions, NO_3^- and CNS^-, differ in effect from the other ions. Li^+ was effective at very low concentrations (0·0005 M.). This

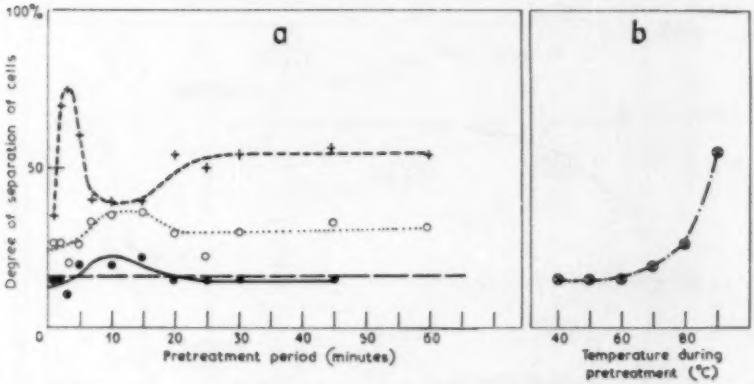


FIG. 6. The effect of pretreatment in distilled water at different temperatures and for various periods on the subsequent separation of cells in pea root tips treated with EDTA. (EDTA 0·1 M., pH 8·0, temp. 25° C., experimental period 17 hours.)

(*a*) Degree of separation as a function of length of pretreatment

●——● Pretreatment at 70° C.
○·····○ Pretreatment at 80° C.
+— — — —+ Pretreatment at 90° C.
— — — — Pretreatment at room temperature.

(*b*) Degree of separation as a function of temperature of pretreatment. (Length of pretreatment period 20 minutes.)

may be due to its H bond breaking action (Kautzman, 1954) although it must be recalled that Li alone, without urea, was not effective in our case. KNO_3 treatment resulted in a plateau, without a sharp optimum, and it was effective even at low concentration. CNS^- inhibited in the entire range of concentrations used. There is at present no explanation available for these results.

The effect of hot water. As hot water is known to be an H-bond breaking agent, its effect on separation of cells was investigated. The hot water was applied as a pretreatment to EDTA; various lengths of pretreatment and various temperatures were studied, and the complete results are given in Fig. 6. The effect of treatment with water at 90° C. could be reversed by treatment with cold water for 1 hour (Table VIII). There is thus a similarity between the effect of hot water and that of urea.

If the effect observed is in fact due to hot water, and not merely due to temperature, then non-aqueous or apolar liquids should show a different

behaviour. The effect of *n*-heptane and xylene treatment was therefore studied. Fig. 7*b* shows that both xylene and heptane were ineffective below 60° C. They became effective between 60–70° C. and a further rise in temperature did not increase their effect. The curve relating degree of separation to length of pretreatment (Fig. 7*a*) shows none of the peculiarities observed for water. Treatment with xylene or heptane increased in effectiveness with

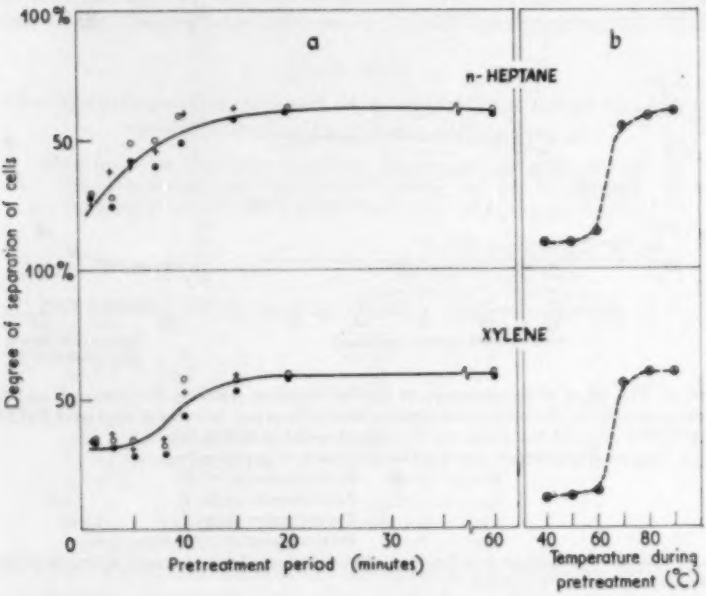


FIG. 7. The effect of (*a*) length of pretreatment with *n*-heptane or xylene at various temperatures (Fig. 7*a*), and (*b*) temperature during a 20-minute pretreatment (Fig. 7*b*) on the separation of cells in pea root tips subsequently treated with EDTA. (EDTA 0·1 M., pH 8·0, temp. 25° C., experimental period 16 hours.)

● Pretreatment at 70° C.
○ Pretreatment at 80° C.
+ Pretreatment at 90° C.

increasing time of application till a constant level was recorded after 20 minutes. Both solvents were entirely similar in their effect. One important difficulty in interpreting the results with the apolar solvents is that no complete elimination of water has been achieved. Nevertheless the fact that, with hot water as with the other solvents, an abrupt change occurred in a narrow range of temperature may indicate the melting of an organized gel.

If water acts as a H-bond breaker its effect should be modified by the ionic strength of the solution. Root tips were pretreated with water at 90° C. in the presence of various concentrations of KCl, for various lengths of time.

Fig. 8 shows essentially three types of response to KCl. Low concentrations up to 0·05 M. KCl prevented the drop in separation which was observed in water alone after 3 minutes. Between 0·1 M. and 0·5 M. the increase in separation was very rapid, a maximum being obtained after 1 minute. The steady level reached corresponded to that in pure water. At higher concentrations

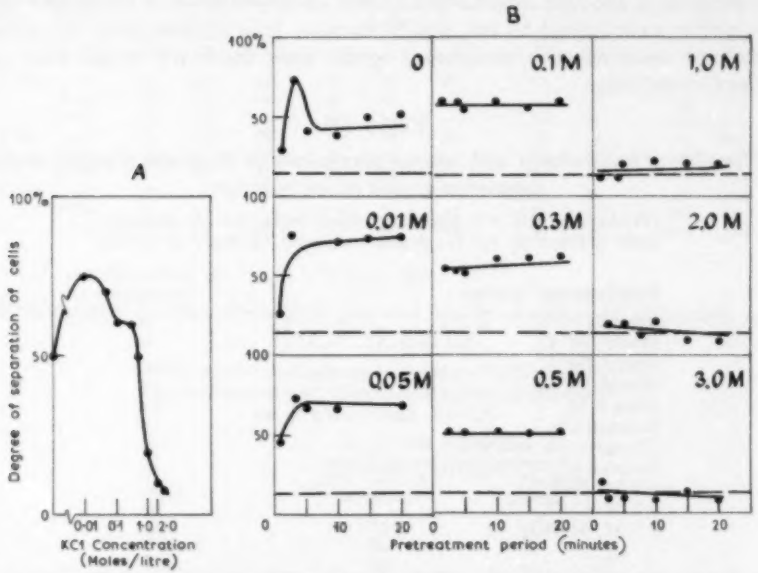


FIG. 8. The effect of concentration of KCl during pretreatment at 90° C. on the relation between length of pretreatment and subsequent separation of cells in pea root tips in EDTA solution. (EDTA 0·1 M., pH 8·0, temp. 25° C., experimental period 15 hours.)
FIG. 8A—Degree of separation (20 minutes' pretreatment) plotted against KCl concentration.
FIG. 8B—Degree of separation plotted against length of pretreatment.
— — — — Pretreatment at room temp.

1·0 M. to 3·0 M. there was an inhibition of the stimulation brought about by hot water. These observations are summed up in Fig. 8A. The results are similar to those obtained for urea except for the fact that in the latter case the inhibition was not observed.

3. *Evidence for the protein nature of the intercellular cement*

The effect of proteolytic enzymes on the separation of cells. Previously it was suggested (Ginzburg, 1958) that a protein component exists in the intercellular cement. It appeared that proteolytic enzymes alone could cause separation of the cells. Further experiments using pure crystalline proteolytic enzymes resulted in only very incomplete separation. Apparently the separation previously reported was due to impurities in the enzyme preparations.

Lindstrom-Lang (1952) suggested that the first step in proteolysis is

denaturation, i.e. destruction of the specific structure of the protein. It thus appeared possible that the protein component in the intercellular cement is stabilized by metal ions and consequently is very resistant to the denaturing action of the enzymes. If, however, denaturing agents such as butanol, urea, thioglycollic acid, or heavy metals were applied before the proteolytic enzymes, separation of the cells might result. Table IX shows some of the results obtained in experiments to test this hypothesis. It is evident from this table that the most effective denaturing agents were the heavy metals such as Zn, Co, and Mn.

TABLE IX

The effect of pretreatment with various denaturants on the action of pepsin on the separation of cells in pea root tips

(*Pepsin 1%, pH 3·5, phosphate-citrate buffer 0·1 M., pretreatment 5 hours at 25° C., pepsin treatment 16 hours at 37° C.*)

Pretreatment solution	% cells separated (in pepsin)
Control	5
Water, 70° C.	0
Water, 80° C.	15
Water, 88° C.	15
Urea 6 M	15
Butanol 5%	7
Thioglycollic acid 1%	20
Butanol 5% + Thioglycollic acid 1%	45
0·8 M. $ZnSO_4$	60
0·8 M. $CoCl_2$	60
0·8 M. $MnSO_2$	60

Recementation of the cells—effect of cations. If separation of the cells by chelating agents is in fact due to removal of a metal ion from a protein structure as suggested above, it should be possible to recement the cells by returning a cation to the structure. This was tested experimentally as follows. Root tips were placed in solutions of EDTA under conditions such as to obtain complete separation. The root tips were then transferred to the salt solution to be tested. After removal from the salt solution they were either examined for recementation at once, or placed in EDTA (*a*) for short periods, 1–2 hours, or (*b*) for longer periods, 12–15 hours. The separation of the cells was then compared with that of controls not treated with salts. This procedure was adopted for the following reason: if recementation results from salt treatment, and if subsequent short immersion in EDTA results in complete separation, then the new 'cement' differs from the original native one. However, if long immersion in EDTA is required to achieve the second separation, this may indicate that the system dealt with is a reversible one.

Table X shows that recementation was obtained by the metal ions used. Similar results were obtained with Fe, Mn, Co, Zn, Ba, &c. It will be seen that for the most part recementation resulted in a behaviour of the tissue very similar to the original behaviour, i.e. separation only after long immersion in

TABLE X

Recementation by salts of separated cells in root tips of peas

The root tips were first treated with 0·1 M. EDTA to effect complete separation of cells, and subsequently transferred to salt solutions; then either examined immediately or treated with EDTA at pH 8·0, 25° C.

Period in salt solution	Subsequent period in EDTA	% separation of cells									
		$CaCl_2$			$MgSO_4$			$CuCl_2$		KCl	
		1·0 M.	10^{-2} M.	10^{-4}M.	1·0 M.	10^{-2} M.	10^{-4} M.	10^{-2} M.	10^{-4} M.	1·0 M.	10^{-2} M.
15 min.	0	12	45	95	25	75	80	—	—	100	100
	2 hrs.	25	50	—	32	75	—	—	—	100	100
	15 hrs.	25	55	—	45	—	—	—	—	100	100
3 hrs.	0	0	15	55	10	12	80	72	50	40	100
	2 hrs.	6	15	65	12	27	—	40	85	100	100
	14 hrs.	18	60	—	30	65	—	65	—	100	100
22 hrs.	0	5	8	25	25	12	—	15	45	40	100
	2 hrs.	—	—	—	—	—	—	25	55	100	100
	14 hrs.	12	35	45	30	40	—	40	55	100	100

TABLE XI

Recementation of separated cells in pea root tips by combinations of nitrates of Fe, Cu, Mg, and Ca

(The cells were initially separated in 0·1 EDTA (85% separation achieved), then transferred to salt solutions for 36 hours, temp. 25° C.)

Salt	% of cells separated	
	$5 \cdot 10^{-6}$ M. salt solution	10^{-5} M. salt solution
Ca	75	65
Mg	80	85
Cu	88	70
Fe	60	60
CaMg	55	62
CaCu	35	50
CaFe	42	52
CuMg	45	60
CuFe	40	45
MgFe	50	50
MgFeCa	35	62
MgFeCu	50	50
FeCuCa	37	35
CuCaMg	—	30
CuMgCaFe	37	30

EDTA. Treatment with 1·0 M. KCl (see Table X) resulted in a very easily reversible recementation, even immersion in water for a short period being effective in subsequent separation of the cells. The action of KCl is thus probably due to the ionic strength of the solution (see p. 87).

Since the results given in Tables II and III suggested that the native cement is stabilized by combinations of cations, it was considered probable that suitable combinations might be more effective in recementing separated cells than cations applied singly. Combinations of some cations which may be present in cell walls were accordingly tested (Ca, Mg, Fe, Cu). The

concentrations (10^{-5}–10^{-6} M.) chosen were such as were ineffective when the cations were applied singly. The results are shown in Table XI. As will be seen, the combination of all four ions was the most effective. Other combinations, however, mainly those of three ions, also give very appreciable recementation. The single ions when applied at concentrations similar to the total concentration in the combinations were almost ineffective.

TABLE XII

The effect of proteolytic enzymes and denaturing agents on the recementing of separated cells in pea roots in solutions of $Ca(NO_3)_2$ and $Cu(NO_3)_2$

(The cells were initially separated in 0·1 M. EDTA solution (75% separation achieved), then treated with the enzymes or denaturing agents, and subsequently with the salts. They were then either examined immediately or transferred to EDTA solution as indicated)

	% of cells separated			
	$Ca(NO_3)_2$ 10^{-2} M. 2nd period in EDTA soln.		$Cu(NO_3)_2$ 10^{-2} M. 2nd period in EDTA soln.	
Treatment	0 min.	30 min.	0 min.	30 min.
Control	5	25	7	30
1 hr. in pepsin	25	65	12	70
1 hr. in cathepsin C.	32	85	15	80
1 hr. in urea 6 M.	30	40	40	75
3 hrs. in thioglycollic acid 1%	42	85	35	55
1 hr. in butanol 5%	25	65	10	50

Recementation of the cells: modification of protein component. If recementation is dependent on the intact structure of the protein component, then modification of the protein by various means, such as proteolytic enzymes or denaturing agents, should prevent recementation. This hypothesis was tested as follows: Root tips were immersed in EDTA under conditions which resulted in full separation. This treatment should have made the protein structure, which is normally resistant, susceptible to attack by proteolytic enzymes owing to the removal of the metal ions. The root tips were then transferred to protein-modifying agents, and subsequently placed in the salt solution. The extent of recementation was then assessed as before. The results are given in Table XII, and show that the capacity to recement is destroyed by all the agents which are known to destroy or modify the structure of the protein molecule. The ability to effect recementation by metal ions is thus dependent on the intact structure of the protein component.

DISCUSSION

The experiments described above were designed to test the supposition that plant cells are bound together by a macromolecular structure stabilized by metal ions. The most likely assumption was that such a macromolecule is a protein, or contains protein. This assumption is borne out by the evidence brought forward above. Agents causing denaturation of proteins facilitated the action of EDTA in separating cells (Table XII, Figs. 3, 4). Solutions which are known to affect H bonds such as urea (Table VII) showed activity

at high concentrations, and, in addition, a negative temperature coefficient was observed for their action. Urea behaves similarly in the denaturation of proteins.

Proteolytic enzymes were able to cause separation of the cells (Table IX), although denaturation was required before they were effective. Moreover, the action of proteolytic enzymes prevented the recementation of the cells (Table XII). All these data are consistent with the view that the molecular structure involved is a protein. This view is particularly strengthened by the observed action of thioglycollic acid (Tables VII, VIII, Fig. 4). Thioglycollic acid is known to act on S—S bonds, causing their reduction. Further, the action of EDTA was greatly affected by the ionic strength of the solution (Fig. 1). It was also dependent on the pH when the tissue was pretreated at various pH's (Fig. 2). Increase in activity at both extremely acid and alkaline pH values was observed. The effects of ionic strength and of pH indicate an ampholytic electrolyte. The only natural macromolecule containing S—S bonds which is an ampholytic polyelectrolyte, which could give rise to all the reactions described above, is probably the protein molecule.

We can therefore conclude that a protein molecule is indeed part of the intercellular cement in pea root tips.

The experiments of Letham (1958) on the separation of various plant tissues and those of Nachmony (personal communication) on liverwort thalli suggest that the occurence of a protein in the intercellular cement of plants is of general occurrence.

In the absence of proteolytic enzymes only chelating agents could cause separation of the tissues. This is clear proof of the participation of metal ions in the intercellular cement. The experiments using combinations of chelating agents, specific for heavy metal ions, with less specific ones (Tables II, III), indicated that more than one type of metal ion is involved. The experiments on the recementation of the cells by combinations of heavy metal ions with calcium and magnesium (Table XI) further supported this view. The intercellular cement appears to contain two types of metal ions, the alkaline earth metals such as calcium, and heavy metals such as iron and copper.

The forces binding plant cells together are relatively strong. A plant tissue is normally stable and resistant to shearing forces. Thus it seems probable that the binding material is of a solid or semi-solid nature. The present work has indicated that the binding material contains proteins in addition to the pectin materials usually assumed to be present. It seems reasonable to suppose that such materials are present in the form of a gel. Such a gel structure characteristically consists of a three-dimensional network interpenetrated by a fluid. The three-dimensional network is built up of cross-linked macromolecules.

It seems probable that the treatments causing separation of the tissue were those causing the breakage of cross-linkages in the gel. Treatments recementing the cells re-established cross-linkages, thus re-forming the gel. From the nature of the agents causing separation of the cells we can draw conclusions as to the nature of the cross-linkages and consequently of the gel itself.

It has been shown that in all cases, except treatment with proteolytic enzymes, removal of the metal ions was a prerequisite for the separation of tissues. Various agents could enhance this effect but could not in the absence of chelates cause separation. Therefore the most important cross-linkage present in the intercellular gel is one containing a polyvalent metal ion.

The linkage involved here is not of the type described by Katchalsky (1954) and Ferry (1948). These authors described polyelectrolyte gels, aggregated by the action of polyvalent ions, which could be liquified either by the addition of monovalent ions or by heat. This was not the case for the gel described here, which was heat stable and resistant to the action of monovalent ions. Thus the metallic cross-linkage involved here cannot be of the coulombic electrostatic type described by Katchalsky.

It seems possible that the metallic cross-linkage in the intercellular cement is of the chelate type. Chelate bonds, in macromolecules in general and in proteins in particular, are characterized by their great stability and specificity (Gurd and Wilcox, 1956). The metal ion in the intercellular cement was only removed by very high concentrations of EDTA, despite the low metal-ion concentration, suggesting a high stability constant. The ions were not removed by feeble chelates such as oxalate or citrate. Recementing of cells was effected only by very specific metal ions or ion combinations. This recalls the second requirement of a chelate bond—its specificity. The chelate bond is usually regarded as being intramolecular, the metal ion being bound by a number of ligands on the same molecule (Martell and Calvin, 1952; Schwarzenbach, 1954). Here the metal is supposed to form cross-linkages *between* molecules and it is therefore suggested that *intermolecular chelation* occurs.

Stable chelates are normally formed when the ligands of the chelating molecule together with metal ions form 5- or 6-membered rings. When chelates consist of 7- or 8-membered rings, the stability constant of the chelate does not differ appreciably from that of a normal complex. When the ring of a chelate contains more than eight members then the stability constant is never greater than that of a complex and may even be less (Schwarzenbach, 1954; Martell and Calvin, 1952).

Chelates are considerably more stable if more than one ring system binds the metal ion. Under such circumstances even unstable rings can be extremely stable. Stabilization can also occur if the ligands binding the metal in the large ring are fixed in position by a rigid structure. In both cases it is essential that there is no distortion in the bonds of the metal ions.

It is difficult to envisage an intermolecular chelate as suggested above consisting of 5- or 6-membered rings. It seems quite possible, however, that such a chelate contains larger rings which are stabilized in some way.

In the chelate whose existence is postulated here, such stabilization can result from the formation of various bonds between neighbouring protein molecules. Such bonds could be S—S bonds and H bonds, for the existence of which evidence has been adduced above. It has been shown (Fig 4) that treatment of tissue with thioglycollate, and to a lesser extent with urea,

resulted in a decrease in the EDTA concentration required to separate the cells. We can interpret these results as showing a decrease in the stability constant of the chelate due to S—S bond breakage, or H bond breakage, or both. Consequently lower EDTA concentrations could extract the ion from the chelate.

Pretreatment of the root tips with high temperatures also resulted in more ready separation of the cells (Figs. 6, 7). The curves in Figs. 6, 7 show a sudden change at a definite temperature. Such changes are suggestive of those occurring at the melting-point of an oriented gel or regular chain arrangement which on melting becomes disorganized or amorphous (Flory, 1956, 1957). If the intermolecular chelate is placed within an oriented gel, then melting of the gel would suddenly decrease the stability constant of the chelate.

Thus it appears that the intercellular cement is in fact a gel containing an intermolecular chelate stabilized by various additional bonds.

A similar structure has been shown to exist in conalbumin (Frankel-Conrat and Fenney, 1950; Gurd and Wilcox, 1956). Conalbumin chelates iron very strongly. Denaturation of the conalbumin abolishes its ability to chelate iron completely. Gurd and Wilcox interpret these results as showing that the ligands chelating iron are widely separated on the molecule. In the natural protein chelation is possible owing to the existence of a configuration giving a rigid structure to the chelate. Denaturation changes the configuration so that the structure is no longer rigid. Stable chelates can thus no longer form. Calvin (1954), Smith *et al.* (1954), and Klotz (1954) all conclude that stable chelate-like bridges can be formed between protein molecules themselves or between protein molecules and smaller molecules.

Not all the results we have described can be explained on the basis of a weakening of the chelate. In certain cases an increase in the rate at which EDTA separates the cells was observed. Such an increase in rate could be the result of an easier access of EDTA to the chelate. EDTA, being a large polyvalent ion, would be expected to penetrate slowly through a cross-linked oriented gel. However, the breaking of some cross-linkages would result in easier penetration of the EDTA through the gel. An increase in the rate of movement of EDTA through the gel would also result from changes in the charge of the gel or in its state of imbibition. Such changes could result from changes in the ionic strength, the presence of various anions, pH, &c., as has been described by Katchalsky (1954) for imbibition in polyelectrolytic gels.

The present work has therefore led to the conclusion that the intercellular cement contains protein molecules linked together by two types of metallic ion. This metallic cross-linkage is chelate in character. The results of Loring and Waritz (1957) with virus, Mazia (1954) with chromosomes, and Green (1956) and Mahler (1956) with the electron transfer particle, could also be interpreted as indicating aggregates of macromolecules, with two or more metal ions, held together as a result of intermolecular chelation in an oriented gel.

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Observations on Phragmosomes

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WITH 10 PLATES

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SUMMARY

Bodies thought to be equivalent to the phragmosomes described by Porter and Caulfield in Onion root tips have been demonstrated in the cytoplasm of *Equisetum* (mother cell), *Pinus* (embryo), and *Anthoceros* (capsule meristem). Their behaviour during stages of mitosis from metaphase to telophase has been traced in *Anthoceros* and they have been shown to persist and perhaps to increase after mitotic growth has ceased. Though commonly associated in a characteristic way with the spindle and cell plate, there is no positive evidence in favour of a causal connexion with either. Bipartition of individual phragmosomes occurs in relation to the equatorial plane of the spindle but multiplication may also occur in other ways at other times. No function can yet be assigned to them nor has a developmental sequence been traceable between phragmosomes and any other cell component. They are therefore provisionally regarded as organelles *sui generis*.

INTRODUCTION

PHRAGMOSOMES were first named as such by Porter and Caulfield (1958) as dark bodies commonly associated in a characteristic manner with the cell plate at telophase in Onion root tips. These bodies were not closely investigated and were thought to disappear at the end of mitosis. The name phragmosome will be used here in a sense which is believed to be the same as that of Porter and Caulfield, although with different material and without rigorous proof of identity. The risk of error in the use of the term is, however, thought to be less sericus than the inconvenience of introducing avoidable new names.

The bodies under discussion are widespread in the plant kingdom. Examples will be quoted from a Gymnosperm, a Pteridophyte, and a Bryophyte, and they are therefore probably present in all main groups of land plants. Identification in algae is less certain, and these will not be included in the present account though there is some reason to think that equivalent bodies may occur among Thallophyta. Structurally the bodies to be referred to as phragmosomes differ characteristically from all other components of the cytoplasm, but their functional significance remains unknown. In this, however, they are not unique, and since at certain stages of cell development they present very striking and peculiar appearances which could easily be confused with quite other cell components, it is highly desirable to elucidate these appearances by a special study as an essential prelude to ultimate understanding.

It should, however, perhaps be explained that this point of view has been forced on the observer in the course of an extended inquiry into the fine structure of one particular plant meristem (*Anthoceros*) which has been in progress for some years and about which several papers have already been completed (Manton, 1960, 1961). The first observations on phragmosomes were obtained by chance and without in any way understanding their nature. Only later, when a measure of familiarity with the material had been achieved, could special attention be directed to them. It is therefore the case that although the *Anthoceros* meristem is the main source of the information to be used here it was not in the first instance specially selected for this purpose. There is therefore no reason to think that *Anthoceros* is more favourable than other plants for study of this particular component, and indeed the limited contact with alternative sources to be quoted below suggests strongly that it might be surpassed if search were made. This is, however, unimportant at the present stage of the inquiry since the main purpose of the investigation is to extend information for the *Anthoceros* meristem to an additional cell component beyond those already examined in the papers cited. The limited digression to other plants is primarily for its comparative value in establishing nomenclature.

MATERIAL AND METHODS

All the material was fixed in the summer or autumn of 1958 with standard methods of osmic fixation (buffered to pH7) and methacrylate embedding. Sections were cut on a Porter–Blum Sorval microtome, using a glass knife, and they were examined on a Siemens Elmiskop I.

The *Anthoceros* meristem consists of the bottom half-millimetre of young capsules of *A. laevis* fixed in late August as described in Manton, 1960. The material is identical with that used previously, and some of the same preparations will be quoted. The *Pinus* material was from young embryos dissected out of their ovules in early August at the stage before differentiation of cotyledons had begun. The *Equisetum* material was from young cones of *E. palustre* fixed in early May. All three plants grow locally in the immediate neighbourhood of Leeds.

OBSERVATIONS

A good preliminary view of phragmosomes in what is believed to be the same general relation to the cell plate as that described by Porter and Caulfield can be obtained from Fig. 1. This is a part of a spore mother cell of *Equisetum palustre* at the end of meiosis in which the cytoplasm between the four nuclei is undergoing the tetrad cleavage. An advancing cell plate enters the field centrally from above. It is still discontinuous at the forward edge, but the disposal of some of the larger cell inclusions on the two sides of the eventual plane of cell cleavage is clearly manifested. The ground substance of the cytoplasm is crowded with vesicles and granules which are not separately labelled, but the larger components include a dictyosome (golgi body) (top right), a proplastid (bottom right), three very dense fat bodies (*F*) and five

phragmosomes (*ph*). Four of the phragmosomes are arranged as opposed pairs on the two sides of the nascent cell plate. Each phragmosome is membrane-bounded with grey contents showing only minor traces of inhomogeneity. Both in the presence of the membrane and the lesser opacity of the contents these bodies contrast with the fat bodies which are intensely electron-opaque and without detectable surface membrane. There is no resemblance to any of the other cell components.

Fig. 2 shows part of the cytoplasm of a relatively quiescent cell of a *Pinus* embryo in which nevertheless mitotic stages were encountered and which is therefore still in the condition of an active meristem. The cytoplasm is virtually non-vacuolated but all the ordinary cell organelles are present. Near the cell wall some rather obscure dark bodies may perhaps be phragmosomes cut in unfavourable attitudes. But an undoubted phragmosome farther away from the cell wall contrasts strikingly both with mitochondria and with a fat body included in the same field. It is again membrane-bounded with grey contents, in this case showing rather stronger signs of inhomogeneity within the phragmosome than in the former example, though the inhomogeneity is of a type which does not lend itself to more detailed description.

A closely similar field, though derived from the bryophyte *Anthoceros*, appears in Fig. 3, with the cell to which it relates contained in the inset picture of Fig. 3*a*. The tissue is the epidermis from the meristematic region at the base of the capsule, which has been cut longitudinally (long axis of the organ transverse to the page). The cells are virtually non-vacuolated, roughly cubical in shape, and oriented on the page with the outer side downwards. The end wall which dominates Fig. 3 (see also Fig. 3*a*) is the product of a recent mitosis, and one phragmosome in a characteristic position near it is indicated by the arrow.

The next five plates illustrate the behaviour of these bodies during the active mitotic stages (metaphase, anaphase, and telophase) in this material. Metaphase, from the innermost layer of the capsule wall, is represented by Figs. 4, 4*a*, and 5. These micrographs, at different magnifications and oriented differently on the page (compare white arrows), nevertheless refer to one and the same cell. Fig. 4 contains the spindle itself cut in an approximately median longitudinal plane which also transects the equatorial plate, while Fig. 5 is tangential. The presence of the equatorial plate is important as evidence of stage; the details (Fig. 4*a*) are otherwise only needed in the present context as a guide to the relative sizes of chromosomes versus other organelles which might be mistaken for them. The most conspicuous of these, notably mitochondria and phragmosomes, crowd the cytoplasm outside the spindle area without invading it (Fig. 4); both are apparently oriented in a definite manner with respect to the long axis of the spindle (see especially Fig. 5) at least in its immediate vicinity. The markedly cylindrical shape of some at least of the phragmosomes at this stage should be noted.

Anaphase in a cell from the same tissue layer as the metaphase just considered is represented by the various micrographs of Figs. 6–9, all of which

refer to one cell, though to several sections of it. Only one of the two polar plates of chromosomes (*chr*) is contained in the field (Figs. 6 and 8) but the plane of section is somewhat tipped and the position of the equator is marked by the layer of phragmosomes external to the original spindle which are here encountered (arrows) in increasing number as the sections become more nearly tangential (see especially Fig. 8). The shapes of individual phragmosomes should be noted (see especially Figs. 6, 7*a*, 7*b*, and 9). They are no longer the smooth cylinders seen at metaphase but instead represent an array of peculiar and striking shapes which can most reasonably be interpreted as stages of bipartition.

During telophase (Figs. 10 and 11) bipartition of the phragmosomes is doubtless completed, because at the end of it (see especially Fig. 11) these bodies have assumed a shape and position relative to the cell plate closely comparable to those previously encountered in relation to a young cell wall at interphase (Fig. 3). It should perhaps be noted that the telophase cell of Figs. 10 and 11 is from the young archesporium and not from the capsule wall, hence its smaller size and more conspicuously embryonic condition.

When mitotic growth has ended, both in the archesporium and in the capsule wall, extension growth on a considerable scale follows. During this process it might be expected that phragmosomes might disappear, but this is very far from being the case. The two final plates are appended in illustration.

In the fully vacuolated condition the cells of the capsule wall are most effectively studied in transverse section. An example of such a section, cut at a level at which spore mother cells have rounded up and are nearing though not yet at their full size, is contained in Fig. 12, the tissue (epidermis) being otherwise comparable with that of Fig. 3*a*. If cut longitudinally the cells at the stage of Fig. 12 would be about four times as long as wide.

Three separate fields from sections comparable to that of Fig. 12 are reproduced in Figs. 13–15, one of which (Fig. 15) is actually included in Fig. 12, while the other two are from another epidermal cell (Fig. 13) and a subepidermal cell (Fig. 14) respectively. The shapes of the phragmosomes in Figs. 13 and 15 should be noted. Both seem likely to refer to a bowl-shaped structure encountered in different planes of section. In spite of a superficial resemblance between Fig. 13 and Fig. 9 the interpretation is therefore not necessarily the same. Whether phragmosomes in vacuolating cells are in fact reproducing we do not know, though they seem to be unmistakably increasing in size.

It should also be noted that in cells at the stage of Figs. 11–13 there is no longer any detectable relation between phragmosomes and particular cell walls. They seem to be present in any part of a cell, including the outer and inner sides of the epidermis, whether the adjacent wall is of relatively recent origin or not.

DISCUSSION

The observations recorded above are manifestly incomplete in several respects since we lack (*a*) chemical information and (*b*) developmental

information of several kinds. The former can only be supplied by a special study devoted to it. With regard to the latter some negative findings should perhaps be reported. Careful attention has been paid at all stages of the investigation to the detection of any signs of continuity between the bodies under study and other cytoplasmic components, but without positive results. It is true that from time to time a phragmosome can be encountered in apparently close association with one or other of the better known cytoplasmic components (mitochondria, rough endoplasmic reticulum, even plastids or vacuoles) but there is no consistent association of such a type as to justify developmental conclusions. While therefore it is difficult to exclude the possibility that these bodies might be derived from or originate others, there is no means at present of proving this; they are therefore best treated as independent organelles until such evidence is forthcoming.

With regard to the more positive findings, the observation that these bodies do not disappear at the close of mitosis is a substantial discrepancy from the previous account by Porter and Caulfield which, if it were certain that their statement was a definitive one, might rightly preclude continued use of their term for the objects described here. It may, however, be doubted whether this is in fact the case. Porter and Caulfield (1958) is a brief communication suggesting a preliminary study. I am therefore still of opinion that we are discussing the same thing.

Be that as it may, the conclusions which seem to follow from the facts recorded here are not quite the same as theirs. Thus there is no direct evidence available from *Anthoceros* to suggest that there is any causal connexion between phragmosomes and the cell plate. It is true that they seem to accumulate preferentially at the surface of the spindle during the act of mitosis and that they become orientated in a manner which is also shown by elongated mitochondria. But whether this is cause or effect is uncertain. The most that can be claimed is that proximity to the equator of the spindle is apparently associated not only with orientation but also with bipartition of the elongated phragmosomes of a kind which has no direct relation to the more complicated happenings within the spindle (notably the bipartition of the chromosomes) but which would nevertheless explain the frequent arrangement of phragmosomes apparently in pairs facing each other on the two sides of the cell plate. If this were all, and I have no evidence to the contrary, this degree of association between phragmosomes and cell plate might be entirely without significance for the formation of the latter.

Nevertheless these observations need not be dismissed as meaningless. They are a manifestation of cytoplasmic happenings outside the spindle itself which are an integral part of the mitotic process and as such deserve study. They may be expected to relate to fairly simple mechanical forces, such as protoplasmic streaming or local transference of water, all doubtless dependent on the presence of the spindle but perhaps more amenable to analysis. The real importance of phragmosomes in the present state of knowledge is therefore probably not in any direct relation that they may or may not have to the cell

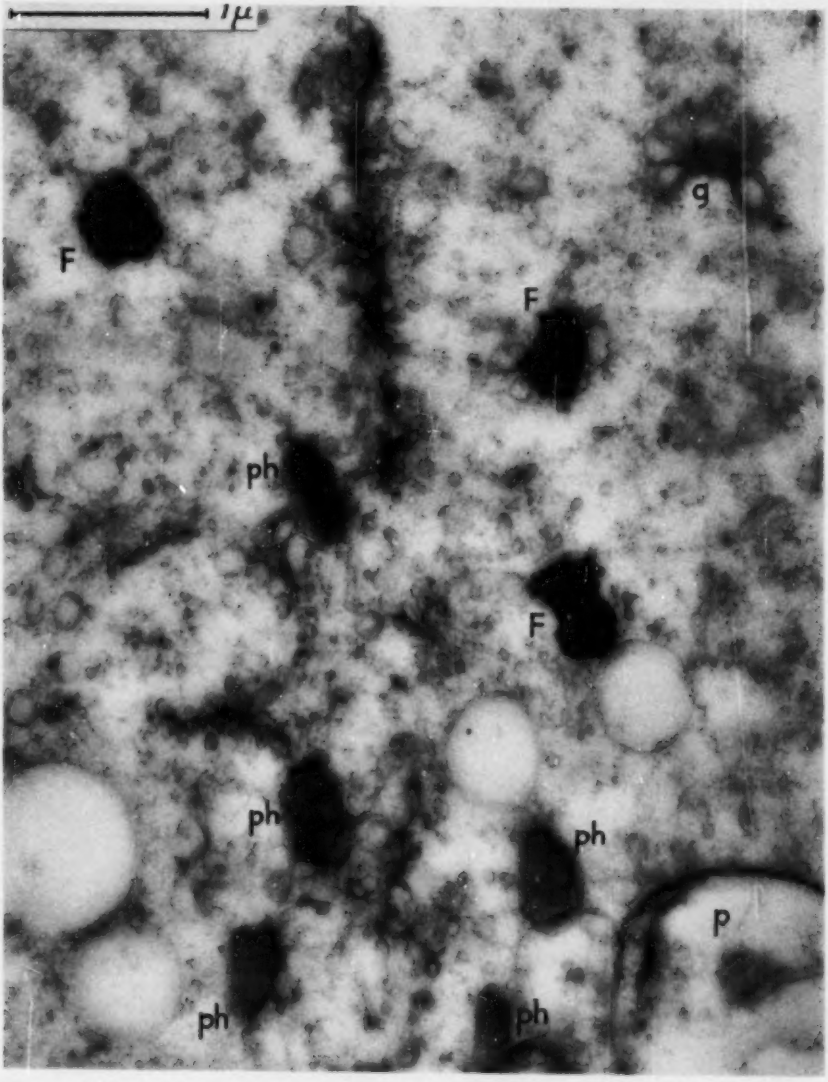
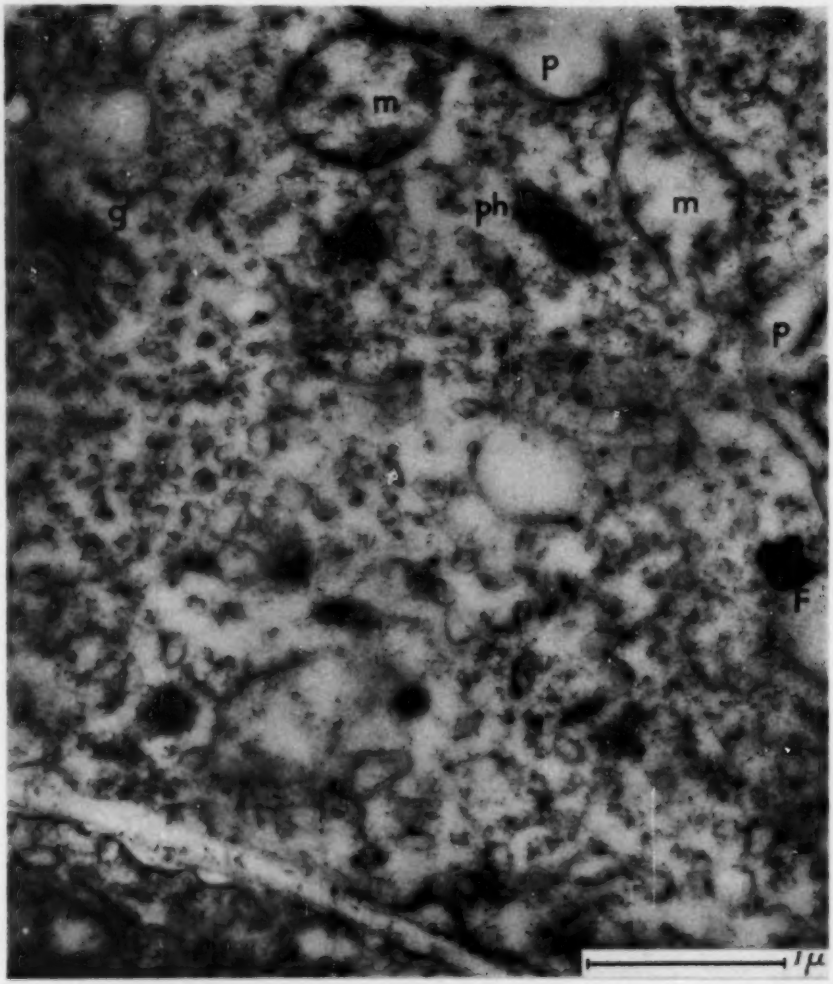


FIG. 1. *Equisetum palustre*: part of a young spore tetrad at telophase of the second meiotic division showing an advancing cell plate centrally, other organelles labelled as in Fig. 2. Micrograph H8970, × 30,000.

I. MANTON—PLATE I

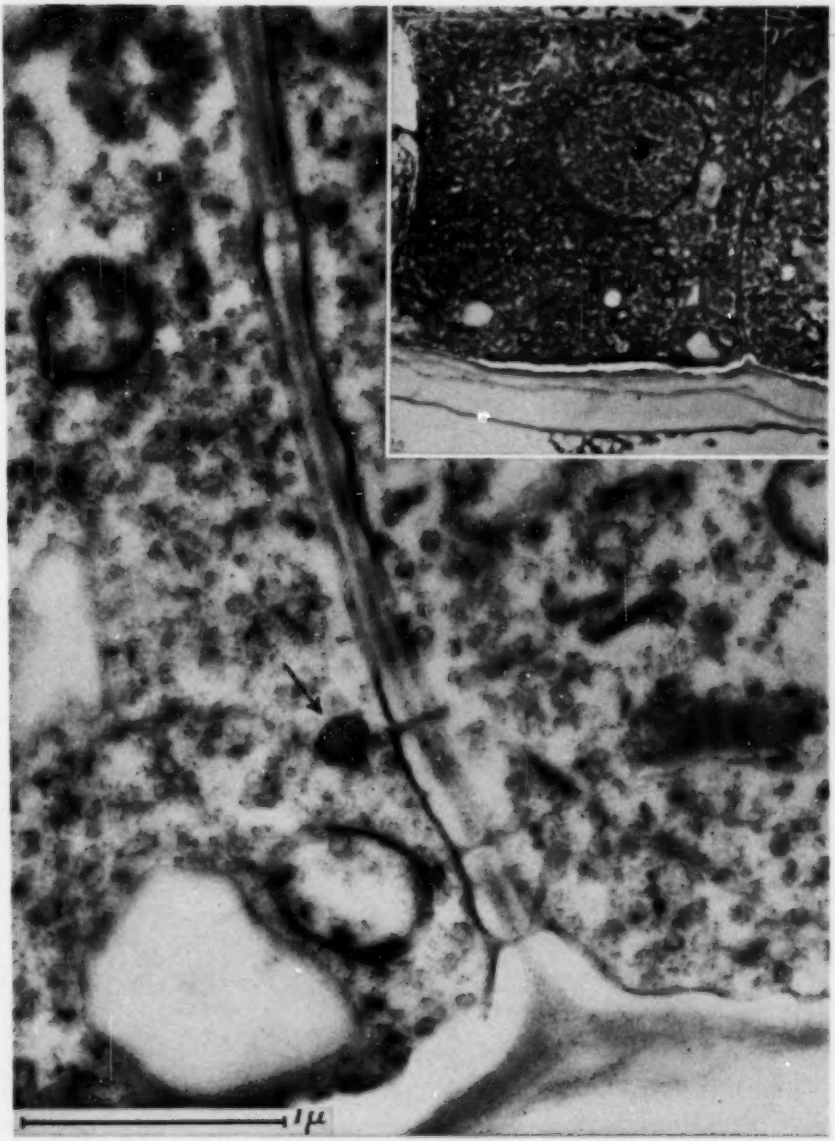


2

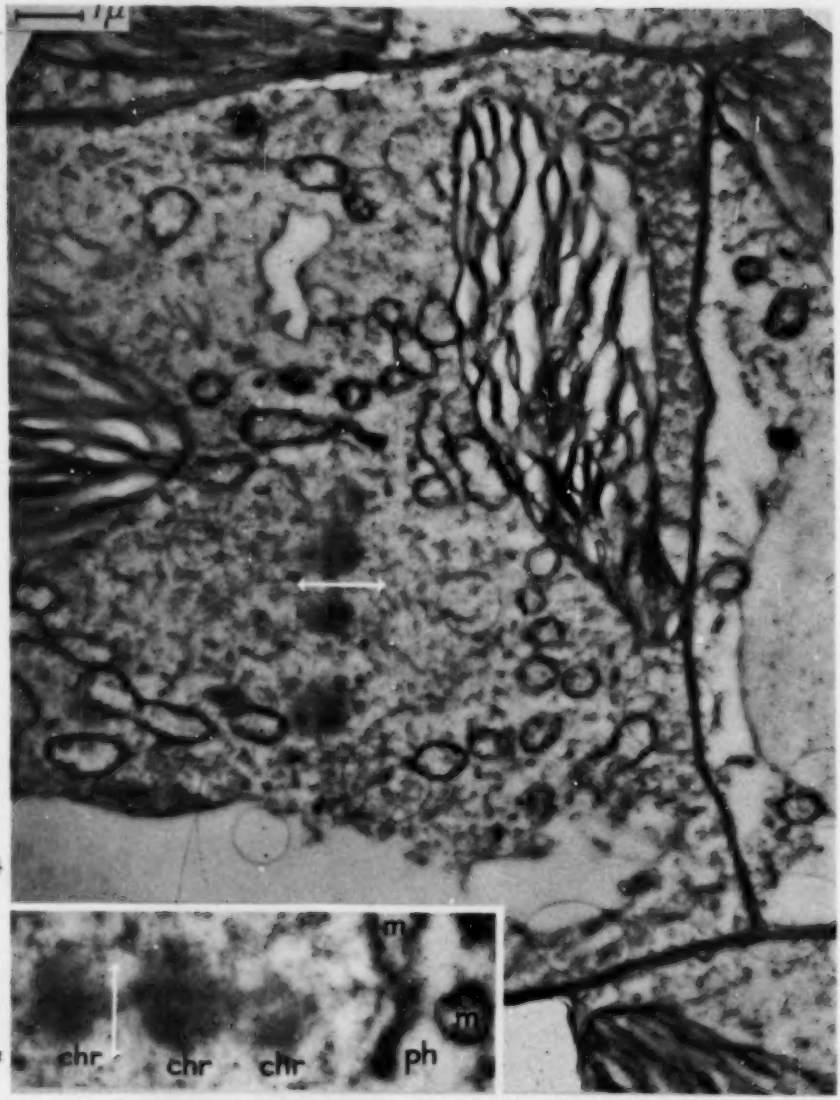
Phragmosome (*ph*), mitochondria (*m*), proplastids (*p*), fat body (*F*), golgi body (*g*).

FIG. 2. *Pinus* embryo: cytoplasm from an interphase cell showing numerous vesicles, granules, and probably traces of other phragmosomes near the cell wall in addition to the named organelles. Micrograph H6920, × 30,000.

I. MANTON—PLATE II

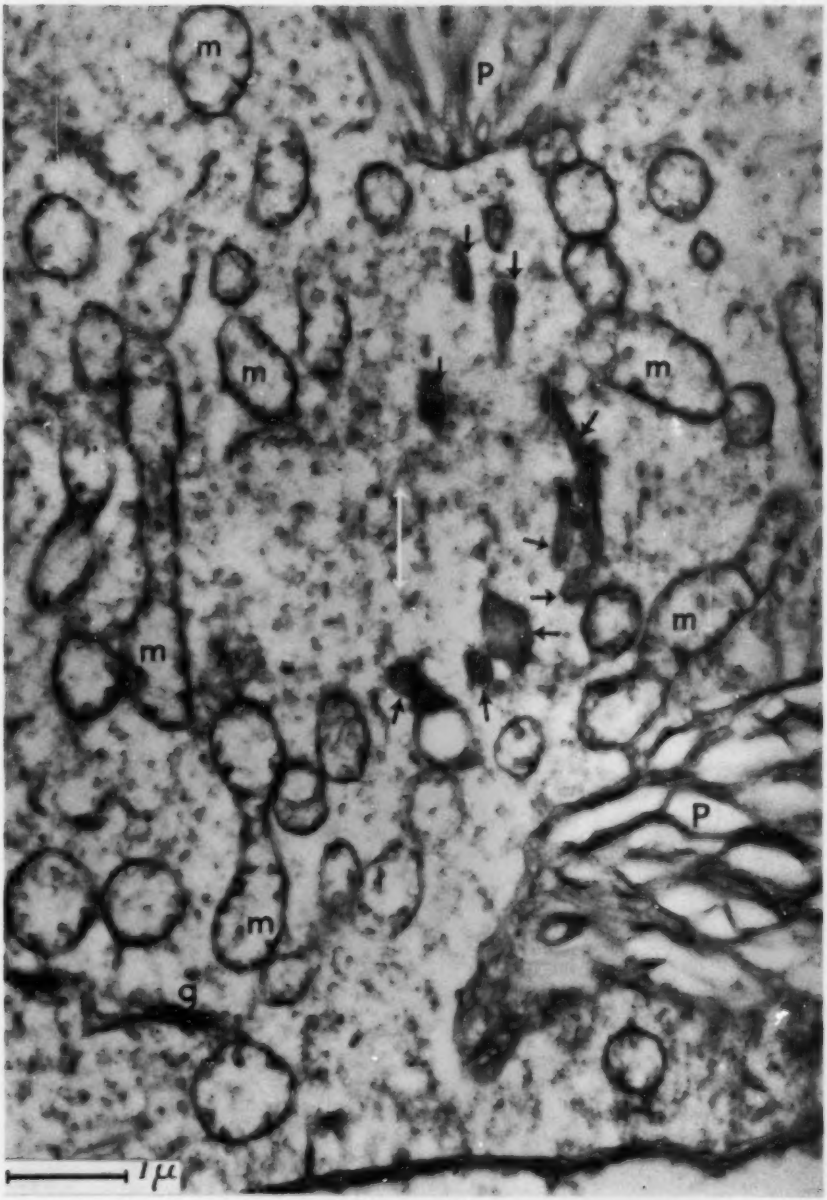


FIGS. 3 and 3*a*. *Anthoceros* meristem: part of the epidermis from a longitudinal section with one cell (outer surface downwards) shown complete in Fig. 3*a* and details from it (right-hand side) in Fig. 3; a phragmosome indicated by the arrow. Micrographs H4704 and 4701; Fig. 3*a* × 5,500, Fig. 3 × 40,000



Chromosomes (*chr*), phragmosomes (*ph*), mitochondria (*m*), direction of long axis (white arrow).

FIG. 4. *Anthoceros* meristem: longitudinal section in the region of the spindle of a cell at metaphase from the innermost layer of the capsule wall. Micrograph H3248, × 10,000. Fig. 4a. more highly magnified part of the equatorial plate in Fig. 4. Micrograph H3249, × 18,000.

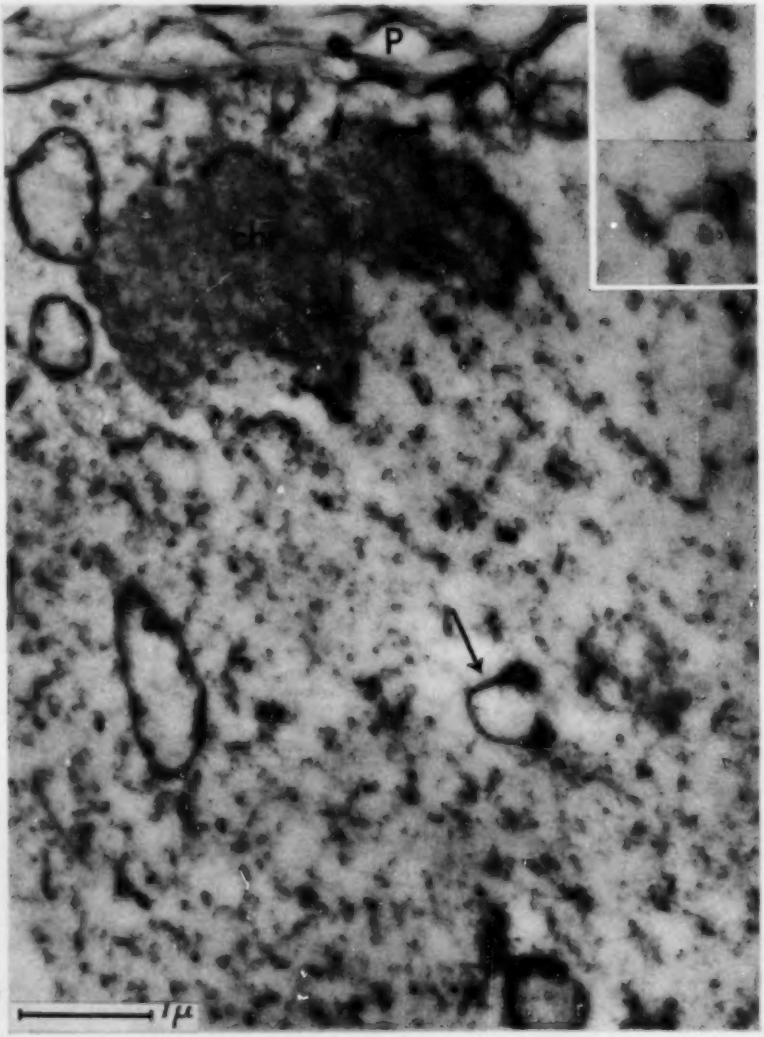


5

Mitochondria (*m*), golgi body (*g*), plastids (*P*), phragmosomes (arrows), direction of long axis (white arrow).

FIG. 5. Another section of the cell of Fig. 4 cut tangentially. Micrograph H3264, × 18,000.

I. MANTON—PLATE V



Chromosomes (*chr*), plastid (*P*), phragmosome (arrow).

FIG. 6. One end of an anaphase from the same cell layer as Figs. 4–5, the long axis slightly tilted. Micrograph H3297, × 20,000.

FIGS. 7*a* and 7*b*. Two successive sections of a phragmosome from another part of the cell (compare Fig. 8, centre). Micrographs H3314 and 3306, × 30,000.

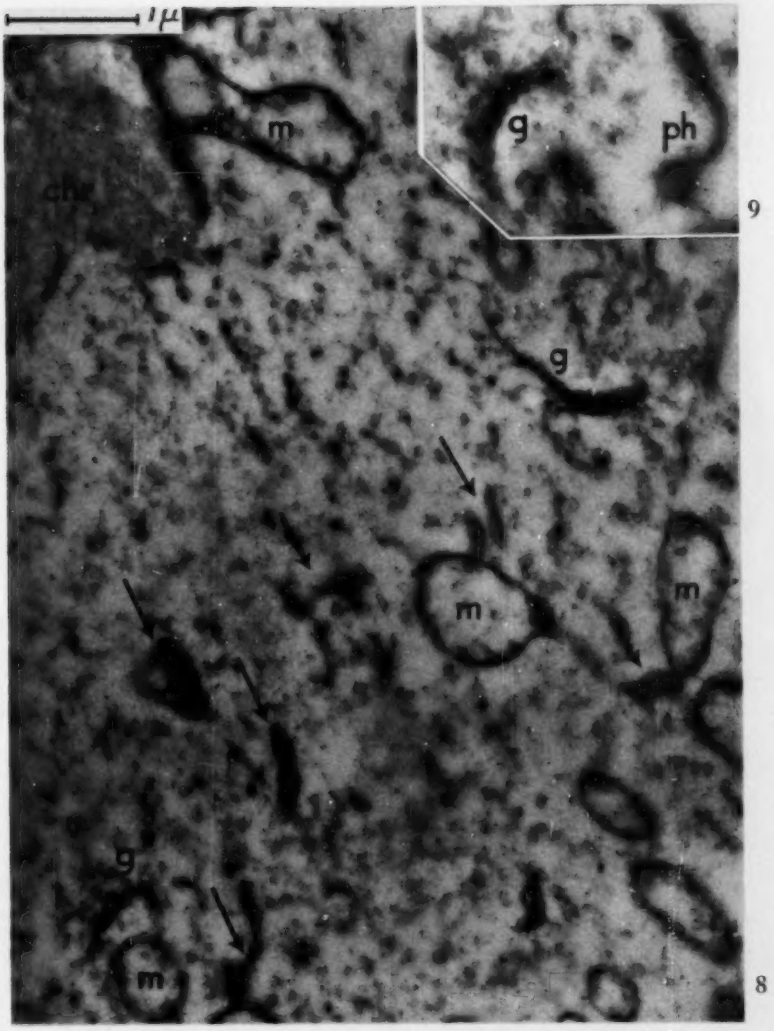
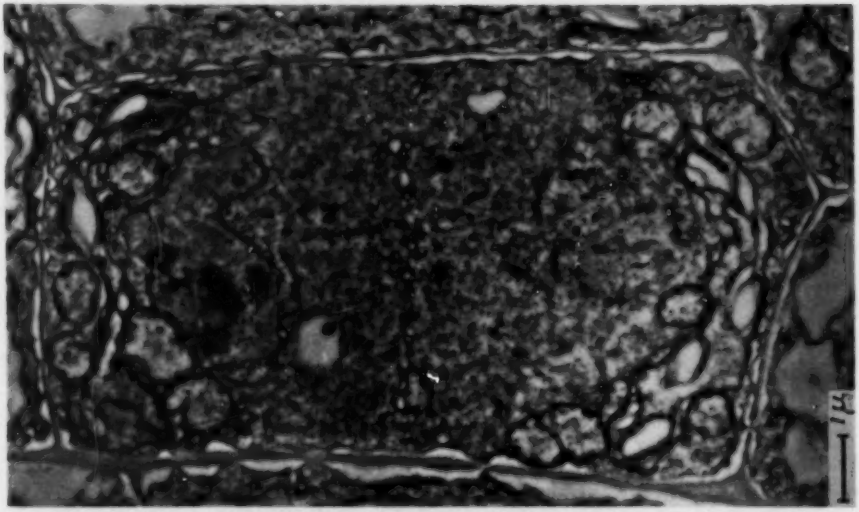


FIG. 8. Another section of the same cell as Fig. 6 cut more tangentially; numerous phragmosomes (arrows) near the level of the equator together with mitochondria (*m*) and golgi bodies (*g*). Micrograph H3306, × 20,000.

FIG. 9. Another section of the phragmosome (*ph*) and gogi body (*g*) in the field of Fig. 8 bottom left. Micrograph H3314, × 30,000.

10



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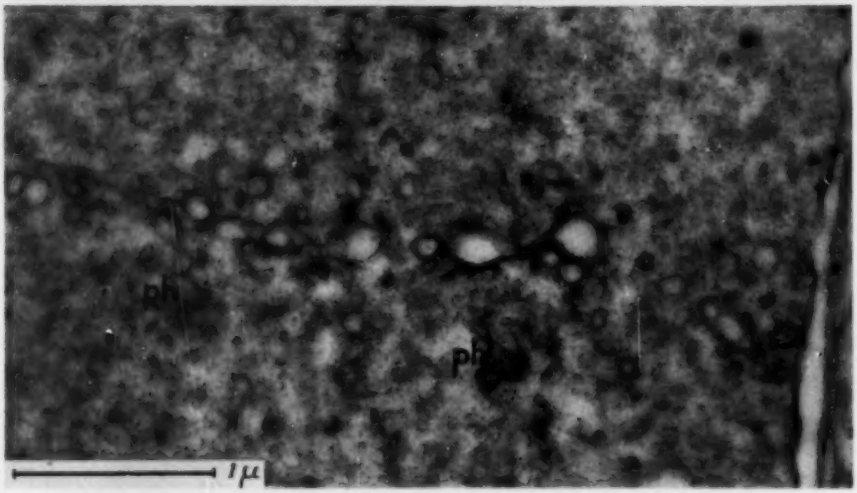

FIG. 10. *Anthoceros* meristem: telophase in an archesporial cell. Micrograph H5840 × 10,000.

FIG. 11. Part of the cell plate from Fig. 10 (upper half) more highly magnified, with two phragmosomes (*ph*) transected near it. Micrograph H5841, × 30,000.

I. MANTON—PLATE VIII

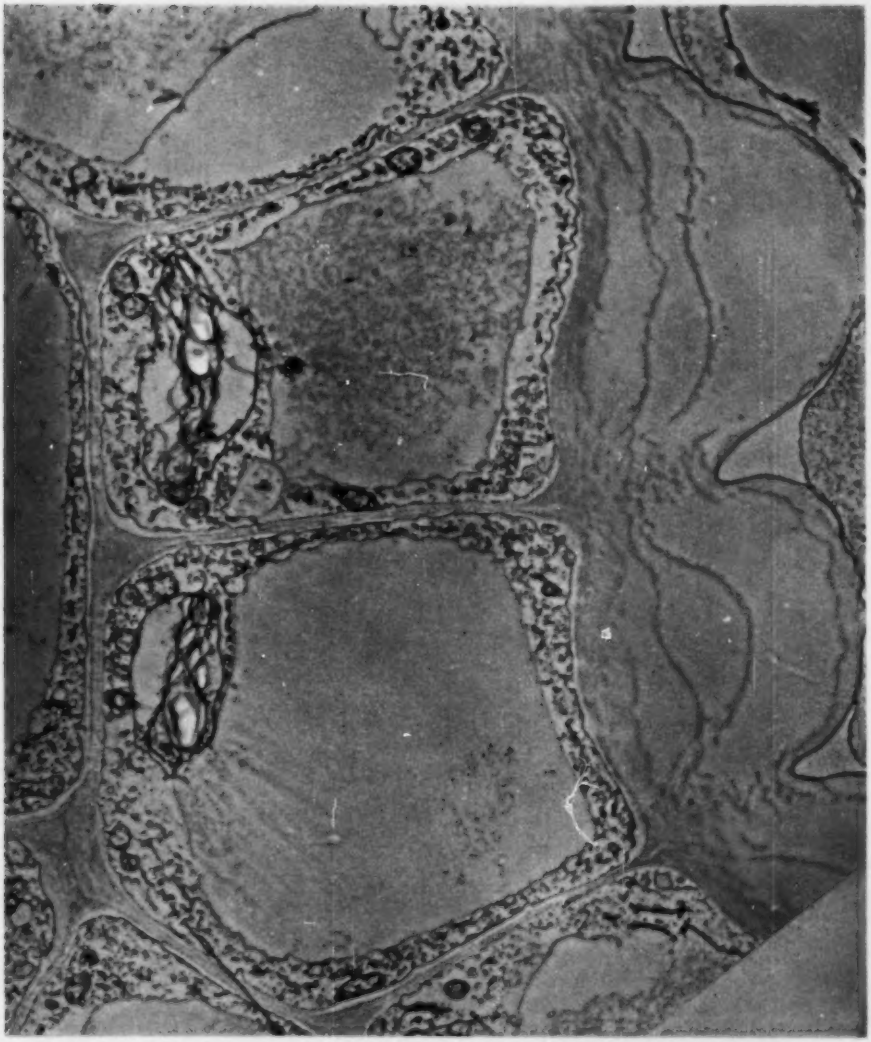

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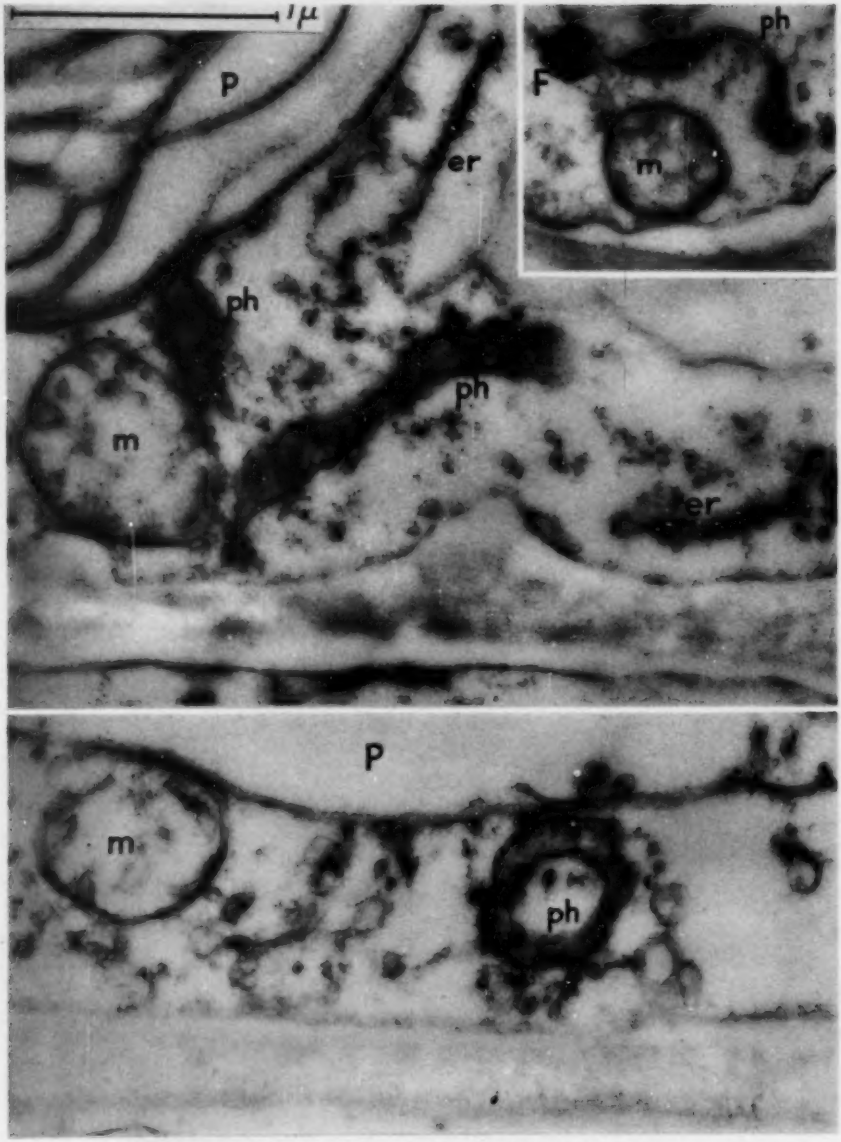
FIG. 12. *Anthoceros* meristem: *TS* through the epidermis of a young capsule undergoing extension growth (contrast with Fig. 3*a*), the outer wall on the right, the subepidermis on the left. Micrograph H8479, × 5,500.



Phragmosomes (*ph*), mitochondria (*m*), fat body (*F*), plastid (*P*), endoplasmic reticulum (*er*).

FIG. 13. Part of an epidermal cell near the outside wall from a section similar to that of Fig. 12. Micrograph H8676, × 30,000.

FIG. 14. Part of a subepidermal cell from a section similar to that of Fig. 12. Micrograph H8534, × 40,000.

FIG. 15. Part of the actual section of Fig. 12 from the cytoplasm between the plastid and the basal wall in the lower epidermal cell. Micrograph H8483, × 40,000.

plate as such, but rather as indicators of a type of cytoplasmic activity which could and should be further studied, not necessarily with the electron microscope.

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The Relationship between the Growth of the Primary Leaf and of the Coleoptile in Seedlings of *Avena* and *Triticum*

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With six Figures in the Text

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SUMMARY

Partial inhibition of extension growth of the primary leaf occurs when whole *Triticum* seedlings are immersed in aerated solutions of IAA but is replaced by growth promotion when sucrose is added to the external solution. In seedlings in which the coleoptile has been excised, IAA increases the growth of the leaf both with and without additional sucrose.

Inhibition of the leaf by moderate concentrations of IAA no longer occurs when the seedling is detached from the endosperm. Sucrose added to the external solution raised the percentage elongation of the coleoptile almost to the level of that attained in intact seedlings without additional carbohydrate. It also enabled the leaf to show a positive growth response with IAA.

The results indicate that in intact seedlings treated with IAA the growth of the primary leaf is markedly diminished owing to diversion of carbohydrate to the coleoptile if the growth of the latter is promoted as a result of the treatment. When the competition of the coleoptile for carbohydrate is diminished or eliminated, acceleration of the growth of the primary leaf by IAA becomes apparent.

In addition to the endogenous rhythm, with a period close to 24 hours, induced in the growth-rate of the coleoptile when seedlings of *Avena* are transferred from red light to darkness, a similar rhythm, with a slightly longer period, is induced in the growth-rate of the primary leaf. This rhythm persists in elongating leaves so long as they remain within the coleoptile. It can be recorded for at least 100 hours in deseeded seedlings.

When intact seedlings of *Avena* are immersed for one hour in relatively high concentrations of IAA and then transferred to distilled water for 18 hours, the elongation of the coleoptile is greater and the inhibition of the leaf is less than when they are transferred to humid air.

Sections of the leaf of *Triticum* showed a slight increase in elongation in concentrations of IAA up to 5 mg./l., but no evidence was obtained that sections of leaf and coleoptile exert any influence on each other's elongation when floated together on solutions of IAA.

INTRODUCTION

It was reported by Bentley and Housley (1954) that when sections of complete coleoptiles of *Avena*, or of primary leaves which had been pushed out of coleoptile sections, were floated on 10 mg./l. solutions of indole-3-acetic acid (IAA) elongation of the primary leaf was promoted. This occurred both

when the leaf remained within the section of the coleoptile and when it was separated from it. On the other hand, it was found by Ball and Dyke (1956) that when intact seedlings of *Avena* were intermittently immersed over a period of one or more hours in IAA or dichlorophenoxyacetic acid (2,4-D), elongation of the coleoptile was promoted, while that of the primary leaf within it was invariably depressed. The chief aim of the present investigation was to determine the reason for the discrepancy.

MATERIAL AND METHODS

Seeds of *Triticum vulgare* var. 'Atle' and *Avena sativa* var. 'Victory' were germinated at 25° C., those of *Avena* being exposed to red light during germination. In the majority of experiments seedlings of *Triticum* were used. These had a stouter coleoptile and were more satisfactory for dissection experiments in which the coleoptile was removed. Seventy-four hours after soaking, seedlings were selected with coleoptiles within the range 17–22 mm., care being taken to choose those in which the apex of the primary leaf was close to the tip of the coleoptile so as to avoid including any in which the rate of elongation of the leaf was abnormally slow.

Whole seedlings were measured both before and after the treatment by placing them alongside a scale on the stage of a binocular microscope, all operations being carried out under red light. The position of the tip of the leaf within the coleoptile could easily be detected when illuminated from below. Lengths were measured from the coleoptilar node to the tip of the coleoptile or of the primary leaf. When sections were used they were cut to an initial length of 10 mm. and at the end of the experiment were measured with the aid of a photographic enlarger.

Owing to variation in the initial length of coleoptiles and leaves, individual seedlings were marked in order that their percentage elongations could be determined. Small labels of aluminium foil were attached to the grain or roots, but this procedure was not feasible in the experiments in which the coleoptiles were removed, as the labels tended to damage the exposed and fragile leaves.

Intact seedlings and those in which the grain or coleoptile had been removed were placed in glass jars containing about 225 ml. distilled water or an appropriate solution. Adequate aeration was secured by subjecting the jars to a to-and-fro movement on a platform shaker at 72 cycles a minute. Sections, on the other hand, were floated on the various liquids.

Except where otherwise stated, treatments with various concentrations of IAA were carried out at 25° C. and lasted for 18–19 hours. Ten or more seedlings were used in each test, which was repeated on a subsequent occasion. Control experiments using distilled water were carried out for each series at the same time.

Special arrangements are described later under the various headings. In certain cases fuller details have been given by Newcombe (1958).

EXPERIMENTAL RESULTS

Intact seedlings—Triticum

1. *IAA alone*

The percentage elongations of the coleoptile and primary leaf during aerated immersion in distilled water and various concentrations of IAA are shown in Fig. 1 (continuous lines).

(*a*) *Coleoptile.* Maximum elongation occurred with 5 mg./l. IAA and was approximately twice that of the control in distilled water. At 30 mg./l. the coleoptiles were flaccid and curled, but their elongation was still slightly above that of the control. In view of the results of Bennet-Clark and Kefford (1954) on the extension growth–time relationship for coleoptile sections in IAA, it is probable that the elongation at 30 mg./l. took place during the first few hours, that is, before the toxic action of the higher concentration became effective.

(*b*) *Leaf.* With concentrations of IAA of 5 mg./l. and above, elongation of the primary leaf was inhibited, inhibition being greatest with 15 mg./l. It then diminished, but a second drop in elongation was recorded at 30 mg./l. At this higher concentration the leaf within the cavity of the coleoptile became curled and partially split so that the decreased elongation was apparently due to a toxic effect of the higher concentration. The inhibition which occurred at lower concentrations cannot be attributed to the same cause, since it became less marked as the concentration was raised from 15 to 25 mg./l. Mer (1957) obtained evidence that the enhanced growth of the mesocotyl in 5 per cent. CO_2 caused diminution in the amount of carbohydrate passing to the coleoptile, the elongation of which was consequently depressed. Similar competition for some nutritional factor coming from the endosperm could account for the fact that promotion of the coleoptile by IAA tends to be accompanied by inhibition of the leaf. On this hypothesis the decreased inhibition of the leaf at 15 to 25 mg./l. can be attributed to the reduced elongation of the coleoptile at these concentrations allowing more of the available food to pass to the leaf. The fact that maximum elongation of the coleoptile, which occurred with 5 mg./l. IAA, was not accompanied by minimum growth of the leaf suggests that the inhibitory effect on the leaf of a lack of food was partially offset by the stimulating action of low concentrations of IAA. On this assumption, the addition of sucrose to the test solution should reduce the uptake by the coleoptile of carbohydrate from the endosperm and enable the promotion of extension growth of the leaf by IAA to become apparent. This possibility was investigated.

2. *IAA in the presence of sucrose*

The results are shown in Fig. 1 (broken lines).

(*a*) *Coleoptile.* Comparison with coleoptiles in distilled water in the same series of tests showed that 2 per cent. sucrose increased the percentage elongation of the coleoptile from $102{\cdot}7 \pm 1{\cdot}6$ to $127 \pm 1{\cdot}7$. IAA at concentrations below 20 mg./l. when added to 2 per cent. sucrose caused greater elongation

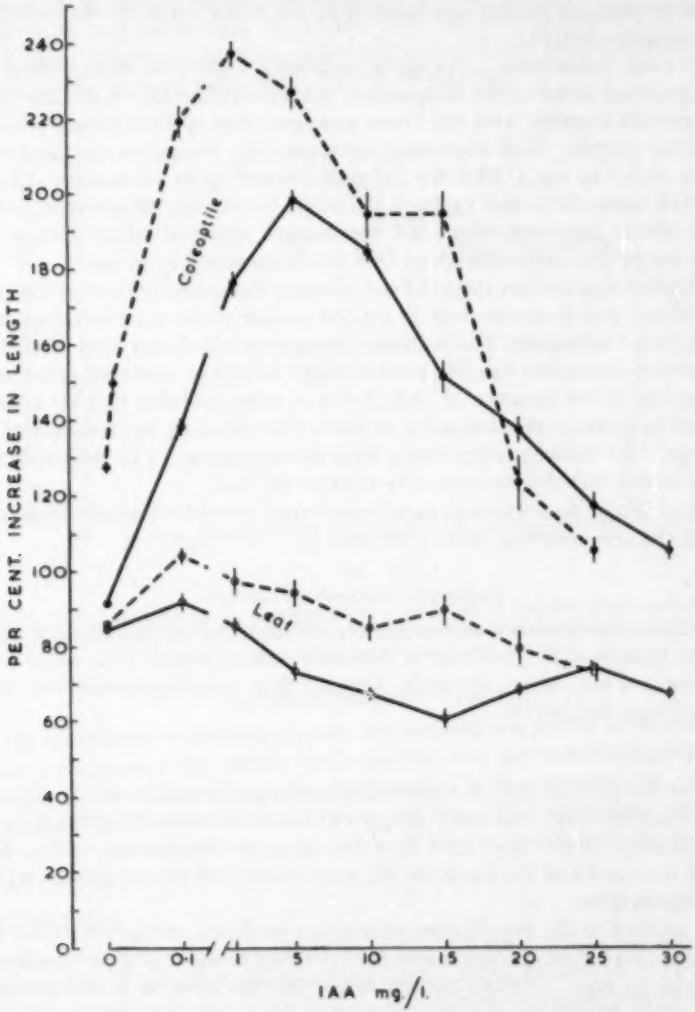

FIG. 1. Percentage elongation of coleoptile and of primary leaf of intact seedlings of *Triticum* in IAA alone (continuous lines) and in IAA + 2 per cent. sucrose (broken lines). Vertical lines at each point represent twice the standard error.

than similar concentrations in distilled water. In the presence of sucrose, maximum elongation—237 per cent.—was obtained with 1 mg./l. IAA, whereas when IAA alone was used the maximum—198 per cent.—occurred with 5 mg./l. IAA. A similar shift to a lower optimum concentration of IAA

in the presence of sucrose was reported by Schneider (1938) for the elongation of coleoptile sections.

(*b*) *Leaf.* Immersion of the whole seedling in 2 per cent. sucrose alone had no significant effect on the elongation of the primary leaf within the coleoptile, but sucrose together with the lower concentrations of IAA did significantly promote growth. With increasing concentration, elongation declined until above about 15 mg./l. IAA the values fell below those for sucrose alone or distilled water. At 20 and 25 mg./l. the leaves became curled and split, so that toxic effects appeared which did not become apparent when sucrose was absent until the concentration of IAA had been raised to 30 mg./l.

The fact that sucrose alone did not promote elongation of the leaf indicates that it was unable to penetrate to the leaf tissues which were surrounded by those of the coleoptile. The increased elongation which occurred when IAA in low concentrations was also present might be due to increased penetration of sucrose in the presence of IAA, but it is more probable that the sucrose helped to promote the elongation of the leaf by reducing the demands of the coleoptile for carbohydrate coming from the endosperm, with the result that more of this carbohydrate was able to enter the leaf.

To obtain further evidence, experiments were carried out on seedlings from which the coleoptile had been removed.

Coleoptile removed—Triticum

In these experiments the coleoptile was detached before the commencement of the treatment by removing a thin slice with a scalpel from either side, starting 3–4 mm. above the node. The two flaps which remained were then turned back and cut off.

1. *IAA alone*

After excision of the coleoptile the percentage elongation of the primary leaf in distilled water was lower ($59{\cdot}4 \pm 0{\cdot}8$) than in intact seedlings ($84{\cdot}6 \pm 0{\cdot}7$). Contributing factors may have been the operational treatment or the effect of the movement of the liquid on the unprotected leaf during growth on the shaking machine.

In contrast to the experiments with intact seedlings, elongation of the leaf was stimulated at all concentrations of IAA below 30 mg./l. (Fig. 2, continuous line). At 30 mg./l. marked curling of the leaf occurred as in the previous experiment. Maximum elongation was at 15 mg./l., whereas with intact seedlings (Fig. 1) maximum inhibition occurred at this concentration.

2. *IAA in the presence of sucrose*

In 2 per cent. sucrose alone the percentage elongation of the leaf was $75{\cdot}9 \pm 2{\cdot}0$, that of the control in distilled water $61{\cdot}5 \pm 2{\cdot}6$. This result contrasts with that obtained with intact seedlings where sucrose had no significant effect on the elongation of the leaf enclosed within the coleoptile. A further increase in elongation of the exposed leaf occurred in mixtures of 2 per cent.

sucrose with IAA in concentrations below 15 mg./l., maximum elongation being with 1–5 mg./l. (Fig. 2, broken line). As with coleoptiles of intact seedlings, maximum elongation of the leaf occurred at a lower concentration of IAA when sucrose was present than in its absence.

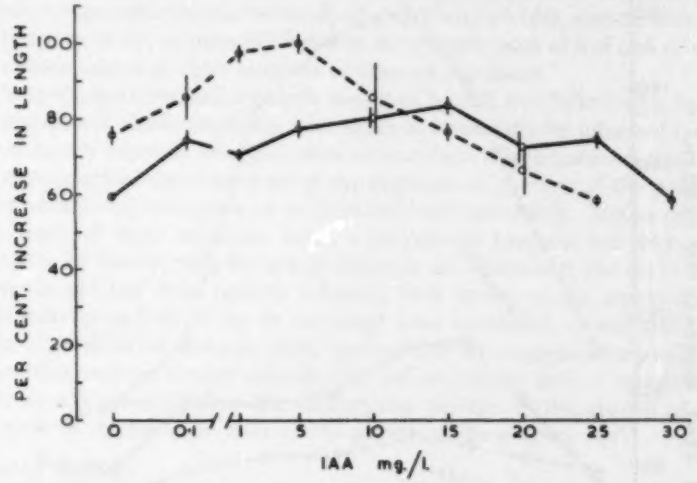

FIG. 2. Percentage elongation of primary leaf of seedlings of *Triticum* after excision of the coleoptile. IAA alone: continuous line; IAA+2 per cent. sucrose: broken line. Vertical lines at each point represent twice the standard error.

Endosperm removed—Triticum

By detaching the grain just before the seedling was placed in the test solution, competition for reserve food material was eliminated. As was to be expected, the control values for elongation of both coleoptile and leaf were considerably less than those for intact seedlings. The percentage elongation of the coleoptile fell from 91·5±0·8 to 55·5±1·0, and that of the leaf from 84·6±0·7 to 47·0±0·7.

1. *IAA alone*

(*a*) *Coleoptile.* At concentrations below 25 mg./l. IAA the percentage elongation of the coleoptile was significantly above that of the control. The maximum occurred at 1–5 mg./l. (Fig. 3), the value being only about half that observed in intact seedlings.

(*b*) *Leaf.* The elongation of the primary leaf was promoted to a very slight extent at low concentrations of IAA. Inhibition occurred with concentrations above 20 mg./l.

2. *IAA in the presence of sucrose*

(*a*) *Coleoptile.* The addition of sucrose to the surrounding liquid increased very considerably the elongation of the coleoptile in the deseeded seedlings.

The percentage elongation in sucrose alone and together with various concentrations of IAA (Fig. 3) was only slightly less than it was with intact seedlings dependent for carbohydrate on the endosperm alone (Fig. 1).

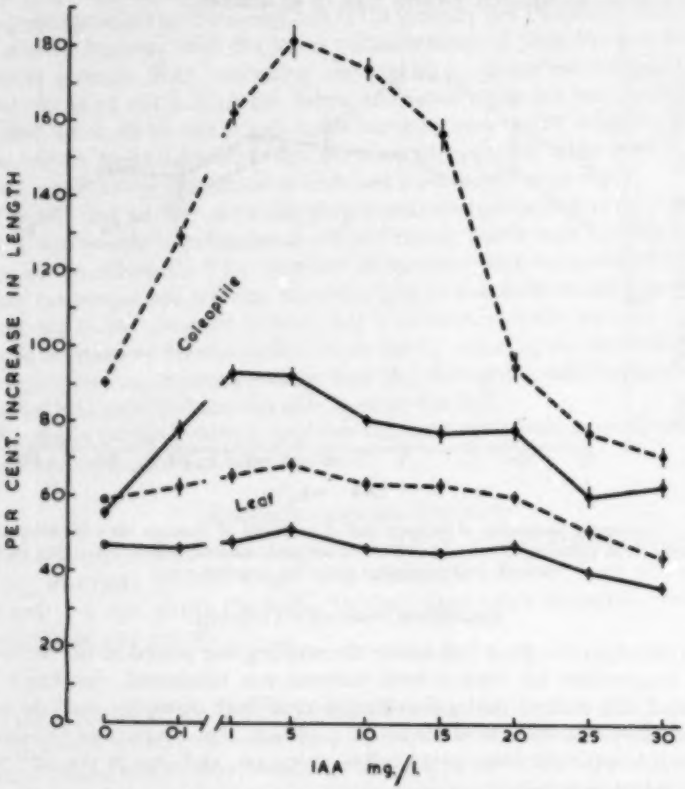

FIG. 3. Percentage elongation of coleoptile and of primary leaf of deseeded seedlings of *Triticum* in IAA alone (continuous lines) and in IAA+2 per cent. sucrose (broken lines). Vertical lines at each point represent twice the standard error.

(*b*) *Leaf.* Elongation of the leaf in deseeded seedlings was raised from 47·5±0·5 per cent. in distilled water to 59·0±0·7 in 2 per cent. sucrose alone. This increase is of the same order as that which occurred in seedlings from which the coleoptile was removed, thus bringing the leaf surface into direct contact with the sucrose solution. Since sucrose apparently did not enter the leaf of intact seedlings one must assume that in the deseeded seedlings in which, also, the leaf was enclosed within the coleoptile, entry occurred through the wound surface produced when the grain was removed. The addition to the sucrose of various concentrations of IAA below 20 mg./l. produced a further small but significant increase in elongation.

Variations in growth-rate during development—Avena

The results of the experiments described so far are based on measurements of the total elongation of coleoptiles and leaves over periods of 18–19 hours during which various treatments were continuously applied. But considerable variation in growth-rate may occur during such long periods, and for a clearer appreciation of the relationship between the growth-rates of leaf and coleoptile measurements at short intervals of time are necessary.

Using the apparatus and methods employed by Ball and Dyke (1954, 1956), photographs of *Avena* seedlings were taken automatically by infra-red radiation at hourly intervals over periods of several days. Slight under-exposure of the photographic negatives enabled the positions of the tips of the primary leaves within the coleoptiles to be detected fairly accurately. Measurements were made of these negatives, using a micrometer eyepiece and a 45-mm. objective. By determining for hourly intervals the distance of the tip of each coleoptile and leaf from suitable reference lines shown on the negative, the mean rates of growth of the 20 seedlings were calculated. *Avena* seedlings rather than those of *Triticum* were used so that the experiments would be comparable to those already described by Ball and Dyke. Also, it was desired to study any possible effect the endogenous rhythm in the growth of the coleoptile of *Avena* might have on the growth of the primary leaf.

1. *Intact seedlings*

Seeds were germinated under red light at 24–25° C. Fifty hours after soaking, 20 seedlings were transferred to the apparatus which was kept at the same temperature. Exposure to red light was continued until the 52nd hour when the light was extinguished. The coleoptiles were then about 10 mm. in length. Photographs of the seedlings were taken by infra-red radiation from the 52nd until the 121st hour.

(*a*) *Coleoptile*. Results are shown in Fig. 4A. As previously reported by Ball and Dyke (1954), the change from light to darkness induces a rhythm in the growth-rate of the coleoptile. The first peak occurred approximately 16 hours after the onset of darkness. A second peak occurred about 23½ hours later. While this peak was developing, the growth-rate did not appear to be affected in any way by the emergence of the leaves.

(*b*) *Leaf*. During the first 19 hours after the transfer to darkness the mean growth-rate of the leaf was fairly similar to that of the coleoptile. Subsequently the growth-rates of both declined, but as the fall in the growth-rate of the coleoptile exceeded that of the leaf, the gap between the tip of the leaf and that of the coleoptile gradually closed. This closure took place in different seedlings at various times between the 76th and 87th hours. After the leaf tip had reached the solid tip of the coleoptile there was a considerable delay ($12{\cdot}5 \pm 3{\cdot}1$ hours) before emergence occurred. During this time the leaf had apparently become slightly compressed, as emergence was accompanied by more rapid elongation, the rate rising to about twice that of the coleoptile during the next 1–2 hours. The growth-rate then fell slightly but soon became

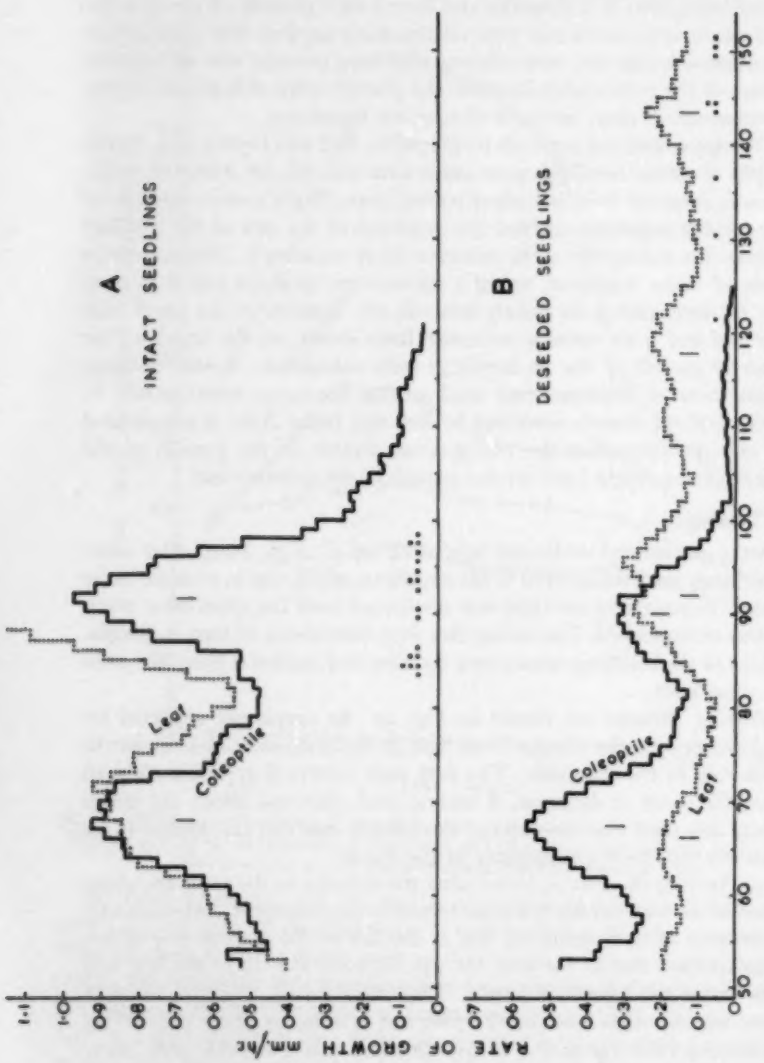

FIG. 4. Mean rates of growth of coleoptiles (continuous lines) and of primary leaf (dotted lines) of seedlings of *Avena* transferred from red light to darkness at the 52nd hour from soaking. *A*: intact seedlings; *B*: seedlings detached from the grain at the 50th hour. The times at which leaves emerged from the coleoptiles is shown by the small squares placed just above the time axis. The horizontal distance between the short vertical lines drawn below each curve represents approximately the period of the rhythm.

much higher than before emergence. As more leaves emerged, the mean growth-rate rose rapidly.

It was reported by Ball, Dyke, and Wilkins (1957) that no significant fluctuations in the growth-rate of the primary leaf could be detected during 24 hours after it had emerged. It now appears that the factors which induce and maintain a rhythm in the growth-rate of the coleoptile of *Avena* have a similar effect on the growth of the primary leaf so long as it is completely enclosed within the coleoptile. In the experiment recorded in Fig. 4A, emergence occurred before a second peak had time to develop.

2. *Deseeded seedlings*

In the next experiment (Fig. 4B) the grain was detached from each seedling as it was put into the apparatus at the 50th hour. As before, the red light was extinguished at the 52nd hour and photographic recording then commenced.

(*a*) *Coleoptile.* Elimination of the food-supply from the endosperm caused a rapid fall in the growth-rate of the coleoptile, followed by a peak which occurred about half an hour earlier than in the experiment with intact seedlings. Later, a second and smaller peak developed; this was about 1½ hours ahead of the control. The fact that starvation is accompanied by an earlier development of the first and second peaks does not necessarily imply that the timekeeping mechanism has been affected by lack of food material. The slight but obvious skewness of the peaks under starvation conditions, owing to the downward slope being steeper than the upward one, has the effect of moving the median line of the peaks in the graph to the left. If the period of the rhythm is estimated by the difference in the times at which the growth-rate curve commences to rise, then the value is obviously very close to 24 hours.

(*b*) *Leaf.* Removal of the endosperm caused a considerable fall in the mean growth-rate of the primary leaf. The value remained less than that of the coleoptile for the next 40 hours. Consequently the gap between leaf tip and coleoptile tip increased during this period and the emergence of the leaves was very much delayed. As shown in Fig. 4B, only 9 leaves had emerged by the time the seedlings were 152 hours old. Four more emerged during the next 18 hours and one more 20 hours later. The remaining 6 had not emerged when the death of the seedlings intervened.

The delay in emergence enabled 4 peaks in the growth-rate curve for the leaf to be detected. Although the peaks are somewhat ill defined it is clear that the period of the rhythm is longer than that of the coleoptile. It appears to be about 25¾ hours. The reason for this increase is still not clear, but it may be noted that Ball and Dyke (1956) found that when the second peak of the growth-rate rhythm of the coleoptile became due at a time when factors inhibiting growth were in operation, it tended to appear a few hours late.

IAA treatment for 1 hour—Triticum *and* Avena

As already mentioned, when intact *Triticum* seedlings were subjected to continuous treatment with IAA for 18–19 hours, maximum inhibition of the

primary leaf occurred with 15 mg./l. With this concentration the mean final length was 71·6 per cent. of the control. A greater degree of inhibition was recorded by Ball and Dyke (1956) when intact *Avena* seedlings were subjected to a much higher concentration of IAA (175 mg./l.) for 1 hour followed by growth in humid air. At the end of 24 hours the elongation of the leaf was reduced to about half that of the control, while that of the coleoptile exceeded the control by about 50 per cent.

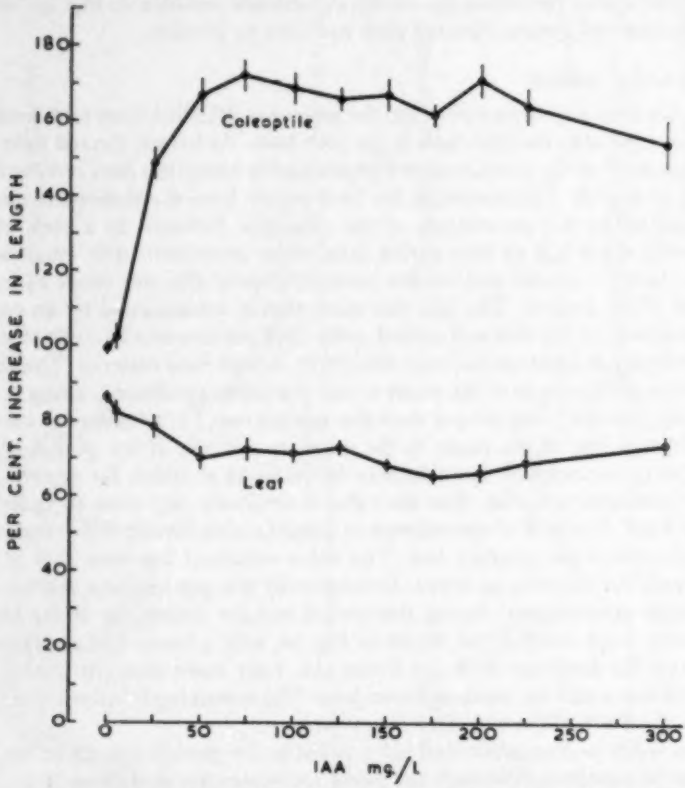

FIG. 5. Percentage elongation of coleoptile and primary leaf of intact seedlings of *Triticum* after 1 hour in IAA followed by 17–18 hours in distilled water. Vertical lines at each point represent twice the standard error.

A series of experiments was therefore carried out on *Triticum* seedlings using the same technique as before, except that the seedlings were transferred from the IAA solution to distilled water at the end of 1 hour. The results are shown in Fig. 5. Elongation of the coleoptile increased with increasing concentration of IAA up to 50–75 mg./l. Beyond this point no further significant change occurred with concentrations up to 200 mg./l., the mean elongation

of the coleoptile over this range being 70 per cent. above the control which was in distilled water throughout. As the concentration of IAA was raised from 0 to 50 mg./l. the increasing elongation of the coleoptile was accompanied by decreasing elongation of the primary leaf. With concentrations above 50 mg./l. there was little or no further change, the elongation of the leaf being then about 20 per cent. less than that of the control.

In similar experiments which were carried out with *Avena* seedlings elongation of the coleoptile reached 125 per cent. above the control value, that of the leaf being 33 per cent. less than the control.

Elongation of the coleoptile is therefore greater and inhibition of the primary leaf is less when intact seedlings are transferred to distilled water after treatment for 1 hour with relatively high concentrations of IAA than when they are kept after treatment in humid air as in the method of Ball and Dyke (1956). The hourly photographic recording employed by Ball and Dyke revealed that promotion of growth of the coleoptile by IAA was followed by inhibition, and their suggestion that this might be due to the production of an inhibitor is supported by the work of Osborne (1958) on *Pisum*.

Recently, Bennet-Clark and Wheeler (1959) have shown that IAA, which is rapidly absorbed by various tissues, including the wheat coleoptile, passes back to a large extent into the surrounding solution within a few hours. Loss of IAA in this way would not have been feasible in the seedlings transferred to humid air, so that the lesser elongation which these showed might be attributed to an inhibitor produced either directly or indirectly from the retained IAA. It must be admitted, however, that there is as yet no direct evidence of the formation of such an inhibitor. Osborne (1958) failed to extract one by diffusion from pea internode sections. She suggested that if an inhibitor is formed within the tissue as a result of treatment with IAA it is possible that it may influence only the growth of the cells in which it is produced. Bennet-Clark and Wheeler (1959) were only able to isolate and identify 3-indolylacetylaspartic acid (IAcAsp) in wheat coleoptiles and pea epicotyls as a result of treatment with IAA. According to Andreae and Good (1955), oat coleoptiles respond to IAcAsp as readily as to IAA in curvature and elongation tests. The cause of the inhibition following a period of treatment with IAA is therefore still uncertain.

Experiments with sections—Triticum

Seedlings were selected with coleoptiles within the range 17–22 mm. 10-mm. sections were cut with a section cutter which also removed the apical 3 mm. of the coleoptile. They were floated on 5 ml. of the appropriate solution and were kept in darkness at 25° C. The lengths were measured at the end of 18 hours. Three experiments were run concurrently. In the first the sections were left intact. In the second the leaf was pushed out of each section and floated on the same solution as the coleoptile. In the third the leaf was removed and floated on a separate solution. Each experiment included a control and one IAA treatment. Ten sections were used in each test.

The results are shown in Fig. 6. The curve showing the response of the coleoptile sections is rather similar to that for the coleoptiles of the deseeded seedlings (Fig. 3). Maximum elongation occurred in both cases at 1–5 mg./l. As in the deseeded seedlings there was a small increase in the elongation of the leaf with concentrations of IAA up to 5 mg./l., but no further significant change up to 20 mg./l.

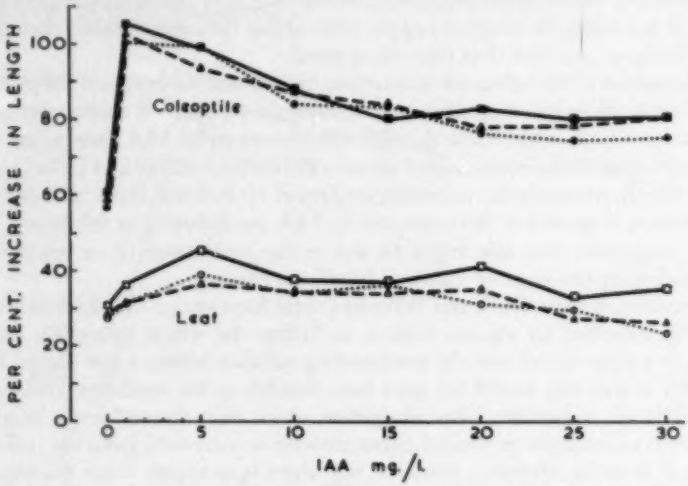

FIG. 6. Percentage elongation of 10-mm. sections of coleoptile and of primary leaf of *Triticum*. Intact sections: continuous lines; leaves removed and floated on same solutions as coleoptiles: broken lines; leaves removed and floated on different solutions from coleoptiles: dotted lines.

Bentley and Housley (1954) found that sections of *Avena* containing the primary leaf showed slightly more elongation in 10 mg./l. IAA than those from which the leaf had been removed. The present experiments with *Triticum* show no significant difference. Nor is there any clear evidence for a beneficial effect exerted by the coleoptile on the leaf as envisaged by Bentley and Housley. The elongation of leaf sections removed and floated on the same solution as the coleoptile sections was not significantly different from that of sections placed in separate dishes. There was a small decrease in the elongation of leaf sections pushed out of the coleoptiles as compared with those left inside, but this decrease may well have been the result of slight injury incurred during the removal.

DISCUSSION

The experiments which have been described show that while the growth of the primary leaf in *Avena* and *Triticum*, like that of the coleoptile, is promoted by IAA, this promotion may be completely replaced by retardation due to diversion of food reserves to the coleoptile as a result of its response to IAA. A somewhat similar picture of competition between mesocotyl and coleoptile

has been revealed by Mer (1957). In the present series of experiments the mesocotyl was either absent or suppressed, and the competition—apart from possible participation by the roots, which was not investigated—was between the coleoptile and primary leaf.

When the grain is detached from the embryo the elongation of the leaf is reduced to a much greater extent than that of the coleoptile (cf. Fig. 4). Probably there was little or no transference of nutrients between the coleoptile and leaf while both were actively growing. But after the growth of the coleoptile had ceased, the mean rate of growth of the leaf showed a slight increase, suggesting that at this stage translocation of some essential metabolite from coleoptile to leaf may have occurred.

The curves in Fig. 4 also reveal that in *Avena* seedlings which have been transferred from red light to darkness an endogenous rhythm in the growth-rate, already known to occur in the coleoptile (Ball and Dyke, 1954), also occurs in the primary leaf. Although the mean rate of growth is low, owing to the removal of the endosperm, the fluctuations in the rate are very marked, the rate at the peaks being at least twice as great as it is between them. The increase in the growth of the leaf occurs at nearly the same time as that of the coleoptile, but this is understandable since in the deseeded seedlings competition for food reserves is eliminated, while in the intact seedlings, where such competition could occur, the rate of growth of the coleoptile was not excessive.

One may postulate that the rates of growth of both coleoptile and leaf can be influenced—probably through a hormone mechanism—by some kind of endogenous timekeeping mechanism or 'clock'. The location of this 'clock' is unknown, but it has been shown by Ball and Dyke (1957) that its efficiency is not affected by removal of the coleoptile tip and it is clear from Fig. 4B that rhythmical fluctuation in the growth-rate of the primary leaf, so long as it is within the coleoptile, continues unabated after the coleoptile has ceased to grow.

A noteworthy feature of the experiments with deseeded seedlings of *Triticum* is that sucrose added to the surrounding liquid, both by itself and together with IAA, restored the elongation of the coleoptile to about the same levels as were recorded with intact seedlings without additional carbohydrate. This contrasts with the observation of Mer (1959) of the failure of sucrose to promote elongation of the coleoptile of *Avena* when supplied through the roots. In his experiments the carbohydrate absorbed in this way was apparently taken up by the mesocotyl, which had not been suppressed by light.

Mer (1959) has also made the interesting observation that when nitrates are supplied to the roots of *Avena* seedlings the growth of both coleoptile and leaves is increased. He attributes this to an increased rate of transfer of reserves from the endosperm to the seedling. It appears from this observation, and from the promotion of coleoptile growth when intact seedlings are supplied with sucrose, that the normal rate of elongation of the coleoptile is limited by the rate of transfer of carbohydrate from the endosperm to the seedling. The leaf, as well as the coleoptile, can make use of additional

carbohydrate provided this has access to the tissues, but in intact seedlings the coleoptile apparently forms a barrier to the passage of sucrose from the external medium to the enclosed leaf.

Under normal conditions there is an equable sharing of the available nutrients between coleoptile and leaf during germination (cf. Fig. 4A). Under the abnormal conditions created by treatment with IAA the greater responsiveness of the coleoptile to auxin enables it to secure a larger proportion of the carbohydrate released from the endosperm, thus imposing a restriction of growth on the primary leaf. Rhythmical fluctuation in the growth-rate, which can be induced in both the coleoptile and the primary leaf, is also a consequence of abnormal conditions, that is, the exposure of the germinating seedling first to illumination and then to darkness. There is at present no reason to think that the rhythm has any beneficial effect on the progress of germination.

ACKNOWLEDGEMENTS

We wish to express our thanks to Professor T. A. Bennet-Clark, F.R.S., for the interest he has taken in this investigation and for kindly providing the necessary facilities. The work was carried out with the aid of a grant from the Agricultural Research Council.

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Midday Closure of Stomata in the Oil Palm *Elaeis guineensis*. Jacq.

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With eight Figures in the Text

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SUMMARY

Under natural climatic conditions in West Africa, midday closure of stomata in 3–6-year-old oil palms occurs only in the latter half of the dry season, when soil moisture is low and air temperatures are high.

Using an infiltration method, attempts were made to determine the climatic factors which affect stomatal closure. The extent of midday closure is related closely to air temperature and shade temperature under the palm, agreeing closely with the findings of Meidner and Heath (1959) on onion, but the aperture–temperature curves are different for watered and unwatered palms in the dry season, the difference being greater at higher temperatures. Watering reduces closure by changing the aperture–temperature relationship. Under natural conditions this effect is usually obscured because of lower temperatures in the dry season following rain. During Harmattan weather, with very low relative humidities, the aperture–temperature relationship broke down.

The results are discussed in relation to current views on midday closure of stomata.

INTRODUCTION

THE oil palm is an extremely important crop plant in West Africa, the produce obtained forming a major part of the exports of Nigeria and Sierra Leone. Despite this, there has been very little work on the plant until recent years, and very little is known of its origin or its distribution in relation to climate. The oil palm is distributed along the West African coast, between latitudes 4° and 12° N., northerly extension of the range being limited, it is believed, by the drier inland climate. It is a matter of general belief that a minimum rainfall of 55–60 in. is required for natural growth and it is said that highest yields are obtained in an annual rainfall of 80–120 in. (Waterston, 1953). The severity of the dry season is likely to be of greater importance than annual rainfall.

The oil palm exhibits some definite symptoms of water stress during the dry season, although the nature of the fibrous leaf, with its thick hypodermis and well-developed cuticle, preclude a recognizable wilting. When a leaf is cut off, the first symptom of water stress is a loss of sheen on the leaflets, which become dull and exhibit some downward rolling. Later a characteristic purple colour develops which rapidly changes to brown as the leaf tissues dry out. There is no wilting. Seedlings suffering from 'Blast', a root disease which reduces effective root absorption, show a rapid development of a dull grey-green colour which is frequently accompanied by an overall yellowing of

leaves (Robertson, 1959). During the dry season there is a progressive decrease in the leaf area of adult palms as a result of abnormal desiccation and death of the older leaves, together with a failure of leaf expansion although primordia are being laid down normally (Broeckmans, 1957).

This work was undertaken to explore the possibility of using stomatal closure as an index of water stress during the dry season. This index was suggested by the work of Maximov and Zernova (1936), and Magness, Degman and Furr (1935).

The stomata, which are borne only on the abaxial surface of the leaves, are semi-xeromorphic, the guard cells being thick-walled with external thickened ridges which are adpressed when the stomata are closed. Subsidiary cells apparently devoid of chlorophyll flank the guard cells and meet between the guard cells and the sub-stomatal cavity when the stomata close. The epidermal cells have a thick cuticle which is completely wettable, and overlie a non-green hypodermis which is more fully developed on the adaxial surface.

A preliminary note on this work has already been published (Rees, 1958).

METHOD AND MATERIALS

Stomatal opening was measured using a modification of the infiltration method of Molisch (1912). In place of the three liquids used by Molisch, a series of isopropanol and water mixtures was prepared of the following composition:

Solution number:	1	2	3	4	5	6	7	8	9	10	11
% isopropanol:	0	10	20	30	40	50	60	70	80	90	100
% water:	100	90	80	70	60	50	40	30	20	10	0

It was found that the solutions could conveniently be carried in small tubes in a test-tube rack.

After placing a drop of solution on the abaxial surface of a leaflet by means of a dropper, penetration of the liquid through open stomata may be observed with the naked eye as scattered dark spots of intercellular flooding. At each reading, the number of the solution of lowest isopropanol content which penetrates the stomata is an index of the degree of closure, the numbers of the solutions forming an inverse scale of stomatal opening. The number of the solution of lowest isopropanol content which was observed to penetrate under a variety of conditions during 4 years' work was No. 4. When the stomata are closed, solution 11 fails to penetrate, and the degree of closure is said to be 12. The method was checked using Lloyd's method of fixing epidermal strips and measuring stomatal pore diameter. The relationship between pore diameter and the number of the penetrating solution is linear, with closure complete when solution 11 fails to penetrate, and the stomata open to 4 μ diameter when solution 4 penetrates. For the purpose of this paper, the numbers 4–12 have been transformed to a scale of percentage closure.

There are some drawbacks to the method; it is not possible to study stomatal movement in the dark, and early morning observations under certain conditions are difficult because the intercellular spaces are frequently water-

logged. On the other hand, permanent injury to leaf tissue was observed only when a solution of high isopropanol content had been applied to a leaflet whose stomata were wide open. In practice, this rarely occurs, although as a precautionary measure no leaflet was treated twice. Application of the solutions to one leaflet had no apparent effect on stomata of neighbouring leaflets. It was found that wet leaflets could be treated if the adhering water was rapidly but gently wiped off before applying the solutions. This treatment did not cause any sudden change in stomatal aperture.

All the results were obtained from a quarter-acre stand of 20 palms which were 3 years old at the start of this investigation in January 1956. Each observation consisted of readings carried out on 6 leaves, 3 on the north and 3 on the south side of a palm. Each set of 3 leaves comprised a young, a middle-aged, and an older, but not senescent, leaf. The young leaves were chosen from the first 3 from the apex, the first fully open leaf being numbered 1. The middle-aged leaves were between the limits of the 10th and 13th leaves, and the older leaves between the 14th and 18th. Each leaf was treated three times, one leaflet being chosen at random from each third of the leaf. The solution was applied to the middle of the abaxial surface of the leaflet and penetration observed. Penetration along the midrib of the leaflet was ignored, as stomata in this position are frequently more open than other stomata. Each sampling provided 18 values for stomatal aperture, the mean of which was used as an estimate of the extent of opening of all the stomata on the palm.

Some of the climatic data used were obtained at the Institute's meteorological station about half a mile from the experimental palms. Evaporation was measured by raised and sunken evaporation tanks, and rainfall was recorded at the palms as well as at the meteorological station. Wet and dry bulb temperatures under the palms were read whenever stomatal observations were made.

At the Main Station of the West African Institute for Oil Palm Research where this work was carried out, the mean annual rainfall is 72 inches, unevenly distributed to give distinct wet and dry seasons, so that 4 months of the year, November, December, January, February, and sometimes March have low rainfall. Mean values over 20 years for rainfall during the dry season are November 2·54 in., December 0·44 in., January 0·46 in., February 1·03 in., and March 3·47 in. These figures are frequently accounted for by one or two storms in a month.

There is a similar seasonal temperature difference, which results from the greater total incident radiation in the dry season, when the cloud cover is far less extensive than in the wet season. The mean daily sunshine in the four dry months is 5·3 hours as compared with 3·5 hours for the remainder of the year. December has 5·7 hours whilst August has 1·9. The mean monthly maximum air temperature reaches a peak of 90·2° F. in March and has a lowest value of 80·8° F. in August.

The soils at W.A.I.F.O.R. are derived from the deep sedimentary Tertiary deposits of the coastal plains of Southern Nigeria. They comprise brownish

topsoil over a great depth of featureless, non-mottled, non-gravelly porous subsoil with coarse sand as the predominant fraction. Clay content increases down the profile, but rarely exceeds 35 per cent. (Vine, 1956). The water-holding capacity is low, field capacity moisture content on oven-dry weight being about 11 per cent. at 3 in. and 17 per cent. at 66 in. and permanent wilting percentage increasing from 5 to 10 per cent. over the same depth.

RESULTS

Results were obtained over a preliminary period of a year, during three dry seasons and part of a wet season.

1. *Preliminary Observations (1956–7)*

During preliminary observations on stomatal movement, the course of mid-day closure was followed throughout a single day in the middle of each month,

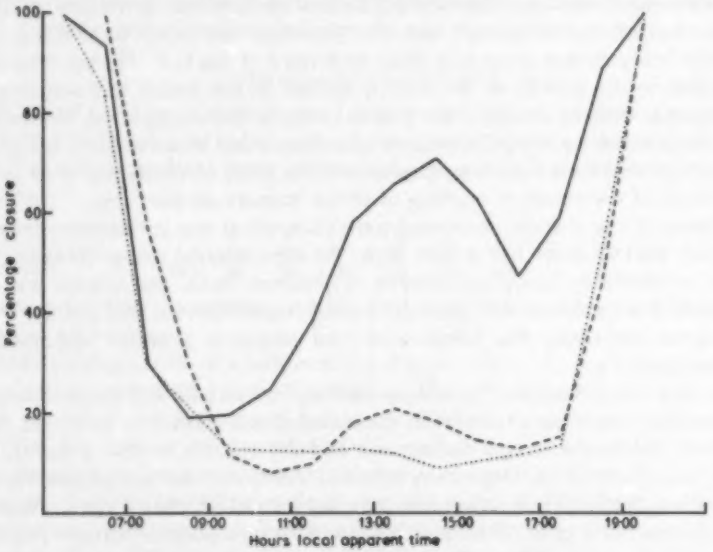

FIG. 1. Typical daily curves of stomatal movement
———, 17.1.56 (dry season)
. . . . . , 19.6.56 (wet season)
– – –, 19.3.56 (intermediate).

starting in January 1956. Typical results are shown in Fig. 1. The course of stomatal movement on 17 January was typical of results obtained on other days in the latter half of the dry season. The early morning rapid opening of stomata continued until about 9.00 hours local apparent time. Closure then set in, the movement gradually reaching a maximum value at about 14.00 hours, after which slight opening occurred until about 16.00 hours when the late afternoon closure began. Closure was completed very shortly after sunset. In

mid-February, essentially the same curve was obtained, although the peak of midday closure was not as marked. This was associated with some cloudy periods near midday.

A month later, following 4.17 in. of rain, the midday closure had almost completely disappeared, and no trace of closure was present in the April, May, and June curves. Observations were discontinued until mid-November. No midday closure was observed then, nor a month later, and spot checks during the middle of the day showed that closure was resumed early in February 1957. The 1956–7 dry season was not nearly as severe as that of the previous year, comparative monthly rainfall figures in inches for these dry seasons and the others considered in this work being shown in Table I.

TABLE I

Dry season rainfall 1955–6 to 1958–9 (in.)

Dry season	*October*	*November*	*December*	*January*	*February*	*March*
1955–6	9·74	2·22	0	0	0·19	7·35
1956–7	15·76	6·35	2·65	0·33	2·71	3·72
1957–8	11·26	0·73	1·15	0·24	0·12	1·67
1958–9	9·09	4·51	0	0·84	1·35	4·95

Stomatal opening integrated over 24 hours as a percentage of maximum possible opening for the period discussed above is shown in Table II. Figures for the duration of daylight are included to show the small range of day-length differences.

TABLE II

Stomatal opening on isolated days in 1956–7

Day	*Duration of day (hours)*	*Opening % of possible in 24 hours*	*Season*	*Rainfall during 30 days previous to observation (in.)*
17 Jan. 1956	11·8	25·78	Late dry	0
17 Feb. 1956	11·9	26·05	,,	0
19 Mar. 1956	12·1	38·87	Early wet	4·17
18 April 1956	12·3	40·50	Wet	9·38
15 May 1956	12·4	44·31	,,	4·39
19 June 1956	12·5	41·96	,,	10·64
6 Nov. 1956	11·9	44·91	,,	10·77
14 Dec. 1956	11·7	45·41	Early dry	4·68
6 Feb. 1957	11·9	33·12	Late dry	0·02

It appears that there are distinct seasonal differences in the extent of midday closure of stomata in the oil palm. For the greater part of the year, during all the wet season and the earlier part of the dry season, the stomata exhibit the normal light-induced opening and closing, remaining open throughout the day. In the latter part of the dry season, midday closure of stomata occurs and its onset may be affected by the severity of the dry season and the date of the last heavy rains of the rainy season.

2. *Dry Season 1956–7*

Following the onset of midday closure in February 1957, daily observations were made of the extent of stomatal closure on one palm computed as the mean of 18 readings taken 2 hours after local apparent noon (this being the time of peak closure).

The observations were continued until mid-May 1957, by which time midday closure had disappeared (Fig. 2).

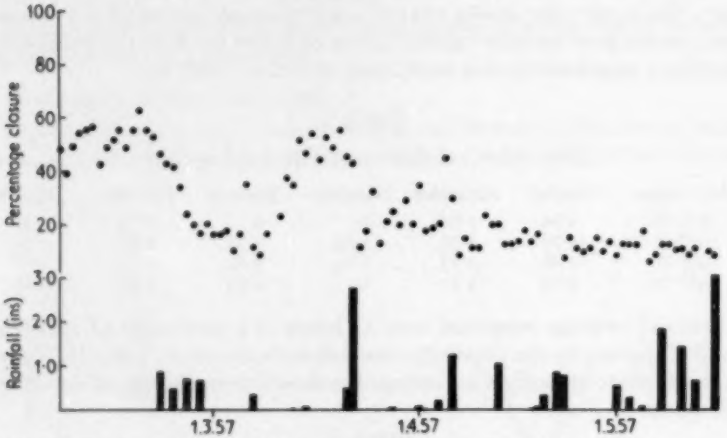

FIG. 2. The daily extent of midday closure of stomata and rainfall during early 1957.

Closure was fairly severe from 6 until 21 or 22 February, when it largely disappeared. There was no rain until 21 February when 0·86 in. fell, followed by 1·85 in. in the next 6 days. Closure was again observed from 11 to 21 March when 0·51 in. rain fell, followed by 2·76 in. the following day. During early April there was some evidence of a resumption of closure, but again it disappeared with the rain. Eventually, as the rains became more frequent, the closure disappeared altogether.

Figures for screen temperature and daily total open-water evaporation were not closely related to the 'wet' and 'dry' conditions, although when the 'wet' and 'dry' periods were separated, a relationship was found between extent of closure and daily evaporation from a raised tank evaporimeter. For convenience of handling the data the period was split up into four as shown in Table III.

3. *Dry Season 1957–8*

Observations on midday closure were resumed during the 1957–8 dry season. Some closure was observed daily from 8 January 1958, although this was not very severe until about 10 February. Daily observations were made until 18 April (see Fig. 3). To obtain further information on the effect of

water in reducing the extent of midday closure, a comparison was made of midday closure in two palms, one of which was amply watered. The comparison extended over 34 days during which time, although rain fell on 14 days, the greatest amount was 0·25 in. and the total 0·89 in. There was no apparent

TABLE III

Midday closure in relation to evaporation, temperature and soil moisture during 1956–7 dry season

Period	*Conditions*	*Dates (incl.) 1957*	*Mean percentage closure*	*Mean daily raised evaporation and screen temperature*	
				in.	°C.
1	Dry	6–22 Feb.	50·7	0·187	32·2
2	Wet	28 Feb.–11 Mar.	17·5	0·187	31·7
3	Dry	14–21 Mar.	49·6	0·167	31·6
4	Wet	24 Mar.–16 May	15·9	0·162	30·8
1+3	Dry	..	50·4	0·180	31·51
2+4	Wet	..	16·3	0·167	30·95

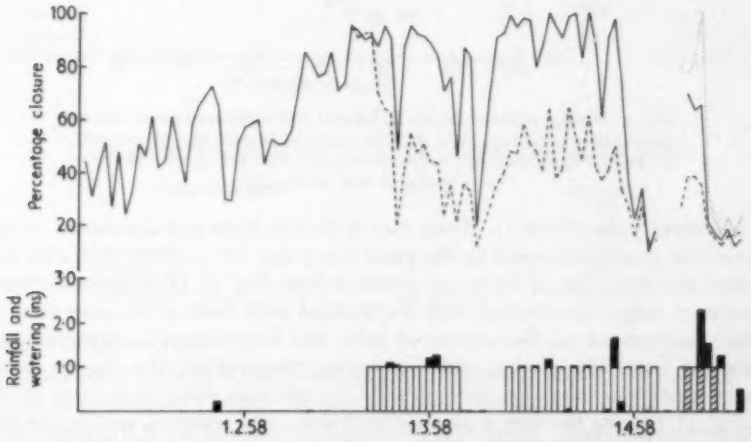

FIG. 3. Daily extent of midday stomatal closure during early 1958. Rainfall is shown as black, irrigation to Palm 1 unshaded, and irrigation to Palm 2 shaded.
– – –, Palm 1; ———, Palm 2; ....., Palm 3.

effect of rain on stomatal closure during the period. The 'dry' palm was watered towards the end of the period to show up any differences not due to treatment. Temperatures at 2 ft. 6 in. above ground level in the shade of the palms were read at the time of the stomatal observations from 25 February.

Unwatered palm—Palm No. 2 (Control). The onset of closure was fairly gradual with some evidence of an effect of a storm at the end of January. Midday stomatal closure was severe from mid-February to the end of March, over 80 per cent. closure being recorded for a greater part of the time and

complete closure on three occasions. On isolated occasions, closure was very much less, and on one day was only 20 per cent. These occasions were all days when the midday temperature was lower than normal. Closure disappeared rapidly at the end of March when 0·89 in. rain fell in 3 days, but was resumed shortly afterwards, only to disappear again following heavy rain. A close relationship was observed between the extent of midday closure and shade temperature, closure increasing very steeply with increasing temperature over the range 32 to 35° C., and being complete or very nearly so at 35 to 36° C. (Fig. 4).

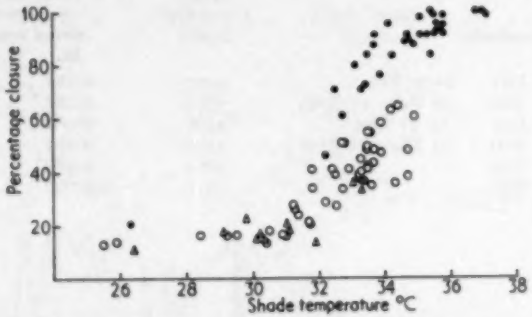

FIG. 4. Midday stomatal closure in watered and unwatered palms and air temperature under the palms. ●, Palm 2 dry conditions (25.2.58–30.3.58); ○, Palm 1 wet conditions (25.2.58–17.4.58); △, Palm 2 wet conditions (31.3.58–3.4.58 and 12–17.4.58).

Watered palm (*Palm 1*). Water was applied to Palm 1 at the rate of 1·0 in. over the ground occupied by the palm every day for 14 days, and after the 22nd day at the rate of 1·0 in. on alternate days (Fig. 3). Over the wider temperature range encountered with the watered palm both at the same time as the observations on the unwatered palm and following rain, stomata again showed increased closure with increasing temperature, the change being very small over the range 26 to 30 or 31° C. and very sharp from 31 to 35° C. (Fig. 4). During the first 3 days before Palm 1 was watered, and on the day of first watering, there was no difference between the extent of midday closure in the two palms, but during the 34 days 25 February to 30 March inclusive, the degree of closure was significantly less in the watered palm. (The period starting 25 February was chosen because figures for shade temperature under the palms were obtained from that date.)

With the appearance of the first heavy rains early in April, the control palm, No. 2, was watered with 1·0 in. on alternate days, and after waiting 3 days to allow the watering to take effect, observations for 6 days were resumed and compared. To obtain comparative values for a palm under natural conditions (control) during this period, Palm 3 was observed from 8 to 18 April (Fig. 3).

Tables IV and V show for the two periods the mean values obtained for stomatal closure and shade temperature. It is clear that the difference in

stomatal closure is due to the treatment and is not a plant difference. During Period 1 there is also a significant difference of shade temperature under the wet and dry palms. The observations on Palm 2 for 9, 10, and 11 April have been omitted from Fig. 4, because of the differences between this palm and the fully watered palm, No. 1. If plotted the points would be at the upper end of the curve for 'wet' palms, although it is clear that the effect of watering did not appear until 11 or 12 April.

TABLE IV

The effect of watering on midday closure of stomata

Palm treatment	*Preliminary 4 days 17.2.58–20.2.58* Percentage closure	*Period 1 34 days 25.2.58–30.3.58* Percentage closure	*Period 2 6 days 12.4.58–17.4.58* Percentage closure
1 unwatered	93·9	..	..
2 unwatered	92·5	85·5	..
1 watered	..	43·1	16·5
2 watered	..	..	16·9
Differences and S.E.	1·4±1·25	42·4±1·7	0·4±0·8

TABLE V

The effect of watering on the midday temperature under palms

Palm treatment	*Period 1* ° C.	*Period 2* ° C.
2 unwatered	34·48	..
1 watered	32·98	30·67
2 watered	..	30·77
Difference	1·50±0·15	0·10±0·48

4. *Wet Season 1958*

Data were collected during the 1958 wet season for the 40-day period, 22 July to 30 August. During the first 11 days there was no rain, as the period corresponded to the brief but variable short dry season. There was no evidence of a shortage of soil moisture. During this time the maximum shade temperature at observation times was 29·5° C. There is a significant positive regression of closure on temperature over the range 23·8 to 29·5° C., the equation of the fitted line being $y = 1{\cdot}251x - 15{\cdot}775$ (Fig. 5).

5. *Dry Season 1958–9*

Daily observations were carried out during the latter part of the wet season and throughout the dry season (from 7 October 1958 to 22 March 1959). The dry season was not so severe as the previous one, rain during January amounting to 1·38 in. The mean monthly maximum temperatures were also lower in the first 3 months of the year. Effects of Harmattan periods, when a very dry north wind blows carrying much suspended dust, were also

observed on three occasions during the dry season. In general, the results confirmed previous observations, but additional information was obtained on soil moisture content in relation to midday closure, and on water absorption following watering of a palm subjected to a long drought period. Attempts were also made to relate closure to relative turgidity of leaves of watered and unwatered palms at dawn and during the midday period.

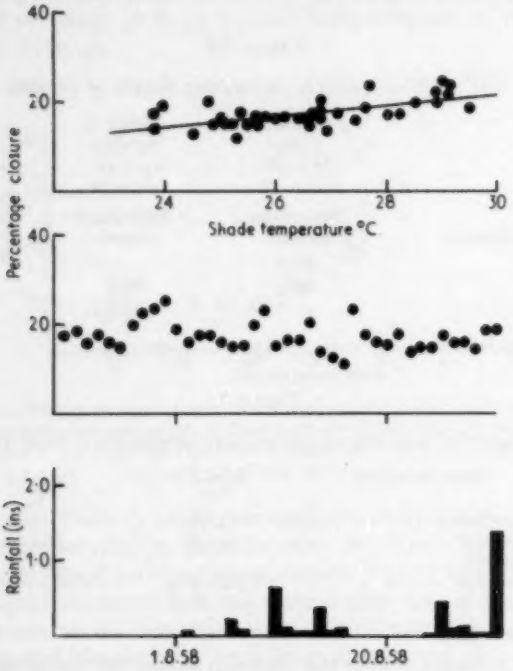

FIG. 5. Wet season observations. Above, temperature–aperture relationship. Slope of line significantly greater than zero, regression $y = 1{\cdot}251x - 15{\cdot}775$. Centre, daily observations of closure, and below, rainfall (in.) during the period.

Soil-moisture content was determined gravimetrically at depths of 3 in., 6 in., 12 in., 18 in., and 30 in. This includes the zone of maximum root density, although roots do penetrate much deeper than this. Once a week, soil samples were taken midway between the base of the palm and the outermost edge of the canopy. It has been shown that the greatest proportion of rainfall reaches the ground near the trunk and at the edges of the canopy (Tinker, 1959). Samples were therefore taken between the area of maximum interception and the outermost edge of the canopy. The data for soil moisture are therefore only approximations of the water content of the soil because of the

effect of the palm crowns in redistributing rainfall. A soil pit was dug to obtain density data for converting moisture content to a volume basis.

Water was not applied as heavily as in the previous dry season, and was given to 2 palms, No. 6, which was watered early in January and throughout the dry period, and No. 5, which was given water towards the end of February. The amounts of water applied are given on Fig. 6.

TABLE VI

Percentage midday stomatal closure and shade temperature for three palms during the 1958–9 dry season

	Preliminary 6 days	*1st period dry*	*2nd period dry*	*3rd period wet*
Palm. Condition	*7–12.1.59*	*5–24.2.59*	*5–13.3.59*	*15–21.3.59*
APERTURE				
4 Wet	21·9	..	..	28·0
Dry	..	64·6	78·1	..
5 Wet	..	..	67·1	26·9
Dry	..	67·1	..	..
6 Wet	23·3	44·0	50·6	28·5
Dry	..	..	..	..
Least significant 1%	3·4	4·5	6·8	4·5
difference 0.1%	..	6·0	9·4	..
DIFFERENCES				
Dry–dry	..	2·5	..	..
Wet–wet	1·4	..	$16·5^{+++}$	0·5, 1·1, 1·6
Wet–dry	..	$20·6^{+++}$ $23·1^{+++}$	$11·0^{+++}$ $27·5^{+++}$	.. ..
TEMPERATURE				
4 Wet	28·63	..	..	31·25
Dry	..	32·46	34·28	..
5 Wet	..	..	34·49	31·21
Dry	..	32·70	..	..
6 Wet	28·78	32·22	33·82	31·01
Dry	..	..	..	..
Least significant 5%	0·26	0·35	0·44	0·76
difference 1%	0·40	0·47	0·60	1·1
DIFFERENCES				
Dry–dry	..	0·24	..	..
Wet–wet	0·15	..	$0·67^{++}$	0·24, 0·20, 0·04
Wet–dry	..	0·24 $0·48^{++}$	0·21 $0·46^{+}$	.. ..

Stomatal closure. Midday closure of stomata of the control palm under natural conditions (No. 4) was first observed on 26 December and extended to 5 January, closure disappearing following 0·61 in. of rain which fell on this date. Closure reappeared on 21 January and extended until 26 February when a further fall of rain affected closure until 5 March. No further extensive midday closure was observed after 13 March. There were three periods when midday closure was obvious, these being separated by two short periods with very reduced closure following rainfall. The extent of closure was not so severe as during the 1957–8 dry season, and only on a few occasions was closure in excess of 80 per cent. observed.

Midday closure was observed in the watered palm (No. 6) on a few occasions, when the extent of closure was comparable with, or only slightly less than, that in the 'dry' palm. This occurred on 21 to 25 January and on 1, and to a lesser extent on 2, 3, and 4 February. These periods correspond with the periods of Harmattan. On 15 and 16 February values for the

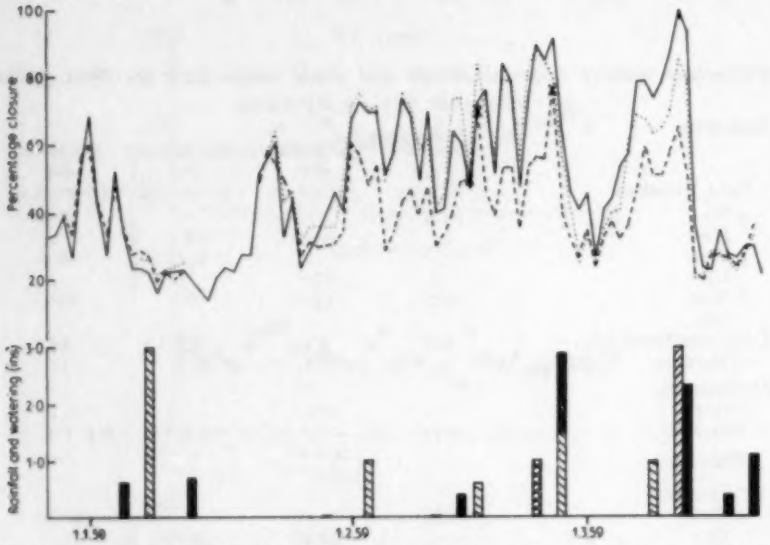

FIG. 6. Daily extent of midday stomatal closure, 1958–9 dry season. Rainfall is shown black, irrigation to Palm 5 shaded ▨, irrigation to Palm 6 shaded ▨. – – –, Palm 6; ———, Palm 4; . . . . . , Palm 5.

extent of midday closure very similar to those of the dry palms were observed for Palm 6. This was also true on 25 February; these days are marked with an 'x' on Fig. 6. In both cases the onset of closure was sudden and disappeared following watering. Throughout the remainer of the dry period, a very marked difference in the extent of midday closure existed between the watered and unwatered palms (Fig. 6).

Application of 1·0 in. of water to Palm 5 on 23 February reduced the extent of closure although the 1·36 in. of rain which fell 3 days later affected Palm 4 similarly. When dry conditions were resumed about 5 March, distinct differences in behaviour occurred, the watered palm showing the least closure, and the dry palm showing most, while Palm 5 (late watered) occupied an intermediate position. Attempts to obtain further information on the behaviour of Palm 5 by giving it 3·0 in. of water on 13 March were foiled by 2·32 in. of rain which fell the following day and reduced closure in all the palms to a very low value. Observations were discontinued after 22 March.

Midday closure and soil moisture. Pronounced midday stomatal closure was fairly closely related to periods of low soil moisture. This was particularly obvious when a period of closure was terminated by rainfall, but the onset of closure in the dry season was less definite. No closure was observed until 26 December, although the moisture content of the upper 30 in. of soil then was as low as that of the next 'dry' period (Fig. 7). The reappearance of closure on 21 January following a 'wet' period was abrupt, because of the coincidence

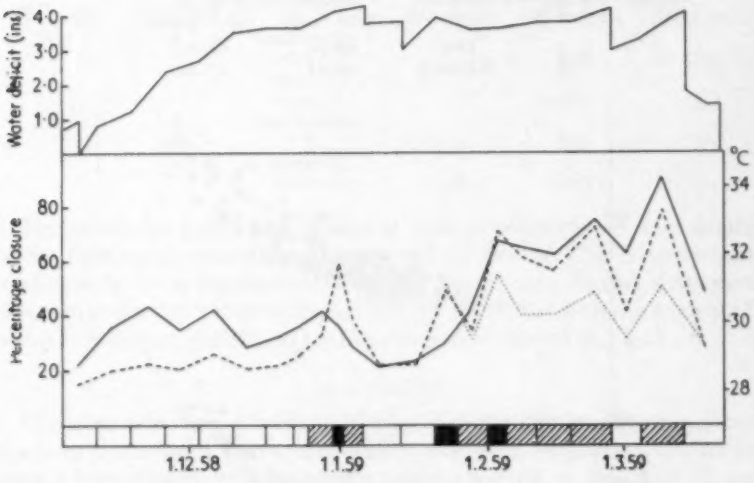

FIG. 7. Soil water deficit in top 30 in. of soil (above) and means of shade temperature and stomatal closure in normal and watered palms (below) during 1958–9 dry season. The periods over which the means are taken are shown below, shaded periods indicate periods of apparent water stress and black periods indicate Harmattan times of very low humidity. ———, temperature; – – –, stomatal closure in normal palm; ....., stomatal closure in watered palm.

of a Harmattan period. Genuine reappearance (in the absence of Harmattan) might have been delayed. Reappearance of closure on 5 March was sudden.

Midday closure and temperature. The relationship between aperture and temperature was as observed in the previous dry season (Fig. 8). This relationship broke down during Harmattan periods, midday closure being exhibited by palms in the 'wet' condition at comparatively low temperatures. Fig. 7 shows weekly means of shade temperature under the control palm and shows how the increased temperatures, which towards the end of the dry season are found associated with low soil moisture, exert a large influence on the extent of closure in both 'wet' and 'dry' palms.

Midday closure and relative turgidity. Relative turgidity was determined for whole leaflets excised from a representative sample of leaves on each palm by the methods described by Weatherley (1951). Sampling was carried out during two periods, the first (7 to 12 January) being a 'wet' period, and the

second (5 to 16 February) being 'dry'. During the first period there was no significant difference between Palms 4 and 6. The results for the second 12-day period are shown in Table VII.

TABLE VII

Relative turgidity at dawn and midday for two palms, one watered and one unwatered, during the dry season. Figures are means of 12 daily observations

Palm	*Condition*	*Relative turgidity at*	
		7.00 hours	*14.00 hours*
4	Dry	99·25	96·89
6	Watered	99·34	97·14

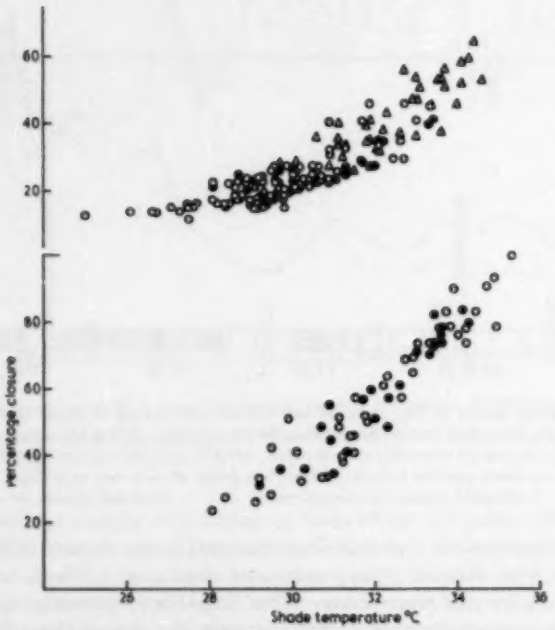

FIG. 8. Relation between shade temperature and percentage stomatal closure during the 1958–9 dry season under wet (above) and dry (below) conditions. ○, Palm 4; ●, Palm 5; △, Palm 6.

In Period 2, Palm 6 has a relative turgidity significantly greater (at 5 per cent.) than that of Palm 4. The magnitude of the difference is, however, very small. In all the samples, relative turgidity at 14.00 hours was very significantly less than at 7.00 hours in both watered and unwatered palms, and in no case was there a 'residual' water deficit at sunrise.

No relationship could be found between relative turgidity and extent of midday closure of stomata.

Midday closure and Harmattan. Harmattan conditions were experienced on the three occasions marked in Fig. 7. The mean values for shade temperature and percentage closure for each period are shown in Table VIII.

TABLE VIII

Temperature under palms and percentage midday stomatal closure during Harmattan periods

			Harmattan period		
Condition	*Palm No.*		*I(2 days)*	*II(5 days)*	*III(4 days)*
Wet	6	mean temp. ° C.	..	29·8	31·9
		mean % closure	..	51·5	55·3
Dry	4 and 5	mean temp. ° C.	29·9	29·6	32·4
		mean % closure	59·6	50·6	71·1

The values for percentage closure at each temperature are considerably higher than those occurring under normal conditions. This is particularly marked at the lower temperatures but not so apparent in the case of the mean observation for dry palms in Period III (71·1 per cent. closure at 32·4° C.) which is only very slightly higher than the values plotted in Fig. 8.

DISCUSSION

The close relationship between midday stomatal closure and temperature has been a consistent feature throughout this work. In general, closure increases very slightly with increasing temperature up to about 30° C. and markedly between 30° C. and 35° C. The temperature index used was shade temperature under the palms, which, in view of the difference between temperatures under watered and unwatered palms, provides a reasonable estimate of leaf temperature.

In recent work, Ansari and Loomis (1959) measured the temperature of a number of different types of leaves under a range of conditions. They conclude that leaves tend to assume air temperature and that transpiration has only a minor effect on leaf temperature in comparison with the effects of radiation and wind, and that wilted leaves show very nearly the same heating and cooling curves as turgid leaves.

Heath and Orchard (1957), Heath and Meidner (1957), and Meidner and Heath (1959) have shown for onion and coffee how midday closure of stomata is affected by Γ, the minimum intercellular space carbon dioxide concentration. Γ is a function of temperature, $\log_e \Gamma$ increasings lightly with leaf temperature up to 30° C. and very steeply above this temperature. Midday closure may therefore be explicable entirely on the basis of leaf temperature changes acting on Γ. The behaviour of oil palm stomata in relation to temperature is very similar to that described by Heath and his co-workers for coffee and onion. The aperture–temperature relationships for palms during

the wet season and for palms watered in the dry season agree closely. The extent of midday closure in palms in the 'dry' condition, when considered separately from the watered palm data, is also closely related to temperature, the relationship being similar to, but not identical with, that found with 'wet' palms.

Watering of palms in the dry season reduces both the extent of midday closure and shade temperatures under them. The temperature reduction was very marked in 1957–8 (1·5° C.) but in the less severe 1958–9 dry season the smaller differences which were not significant in all cases may be related to the relative severity of the dry seasons. Temperature differences cannot account for the different aperture–temperature curves of the watered and unwatered palms, which must be ascribed to an effect of water strain. Watering a 'dry 'palm causes, after a lag of 2 or 3 days, a decrease in midday stomatal closure. Drying causes a rapid change in the extent of closure in a 'wet' palm, the change being, in effect, a shift from one aperture–temperature curve to another.

There are *two* factors which influence the occurrence and extent of midday stomatal closure in the oil palm, the first being temperature and the second water strain. Under natural conditions, these relationships are not so clear. At low temperatures in the 'dry' condition (which is a rare occurrence) the extent of closure is negligible; at the higher temperature range the water effect is most marked, complete closure occurring at about 35° C. The change from the 'wet' to the 'dry' condition is not clear early in the dry season because ambient temperatures are usually too low to show differences between watered and unwatered palms (see Fig. 3, January 1957). Reduced closure following rain during the dry season may also have two causes, since the reduction in ambient temperature which occurs may persist for a few days, thus masking the true water effect on aperture which can only be clearly detected at the upper temperature range.

Watering towards the end of the dry season failed to reduce the extent of closure from the 'dry' to the 'wet' values (Fig. 6). This is believed to be an effect of water strain resulting from the death of a large proportion of the root system, particularly that in the upper layers of soil. This was confirmed qualitatively when a large number of tertiary and quaternary roots were found to be dead. This fact may also explain the significant difference in temperature under the 'dry' palm and the 'fully watered' palm following rain at the end of the 1958–9 dry season (Table V), for Palm 4 would be expected to have impeded water absorption as a result of root damage, leading to a lower transpiration rate, and higher leaf temperatures.

The relative turgidity values obtained during this investigation were never less than 96 at 14.00 hours local apparent time. The figures are very much higher than those observed by Weatherley (1951) and Hutchinson, Manning, and Farbrother (1958) for cotton, and by Werner (1954) on potato. The very small, though significant, difference between a watered and unwatered palm in the dry season when a big difference exists in the extent of stomatal closure,

suggests that relative turgidity of oil palm leaves is not such a sensitive measure of water stress as it is in softer-leaved plants, probably because of a greater proportion of lignified skeletal tissue. Weatherley (1951) describes a critical moisture content, below which relative turgidity is related to soil moisture as well as atmospheric conditions. This is probably the same phenomenon as that described by Penman (1956) as a fairly sharp transition between a uniform maximum transpiration rate and a lower rate when water is limiting. In the oil palm this is best observed as a stomatal effect. When soil moisture is non-limiting, there is some closure, especially at higher temperatures, but when soil moisture falls below a critical level (Fig. 6, points marked '**x**') there is a suddenly increased stomatal closure which disappears following watering.

Heath and Meidner (1958), using detached wheat leaves, showed that Γ is affected by water strain produced by placing their sheaths in mannitol solutions and by withdrawing the water-supply. Scarth and Shaw (1951) showed for *Pelargonium* that stomatal closure induced by mild water deficit may be largely prevented by supplying air free of carbon dioxide. An increase in hydroactive closure caused by carbon dioxide was demonstrated by Stålfelt (1959) although the mechanism relating water-supply to carbon dioxide is not clear. The results of Heath and Meidner (1958) suggest the existence in wheat of a closing response of stomata to water-strain operated through carbon dioxide. Such a mechanism may operate in the oil palm in addition to the more clearly demonstrated temperature effect.

The only closure observed at comparatively low temperatures was during Harmattan periods of very low relative humidity. Similar effects of very dry air have been observed in Israel (Oppenheimer and Mendel, 1939), in northern Australia (Slatyer, 1956), and under laboratory conditions (Heath and Milthorpe, 1950). These may be related to water stress induced by very high potential evapotranspiration rates; it is known that when this exceeds 4–5 mm. per day, adverse deficits develop even if the soil is at field capacity (Milthorpe, 1960).

ACKNOWLEDGEMENTS

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The Chemical and Mechanical State of the Cell Wall of Pea Root Tips

I. PRELIMINARY OBSERVATIONS

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With four Figures in the Text

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SUMMARY

The separation of pea root-tip cells after treatment with acid was studied by the use of a mechanical device that squashed the tissues under a constant load. The increase in area of the tissue as a result of the squashing was used as a quantitative measure of the cohesive forces that held the cells together.

Interruption of the tissue respiration prior to the testing, by incubation under nitrogen for a period of 15 minutes or longer, led to a substantial increase in the acid-resistant mechanical strength, as was also the case if respiratory inhibitors were added to root tips during incubation under air. It was also found that this response varies in serial segments of the root, being greatest in the actively growing regions.

The nature of this change in the state of the tissue is discussed, and the conclusion is drawn that in the natural state of the tissues a mechanism linked to the respiratory processes must be responsible for maintaining the tissues in a state susceptible to treatment with acid.

INTRODUCTION

THE investigation reported here involves a preliminary examination of the relation between respiration and the acid lability of the wall component that binds cells together. It arose from a chance observation that root fragments that have been exposed to anaerobic conditions are less readily disrupted after the acid treatment of the Feulgen procedure than are others that have been exposed to air. This observation suggests that the state of the intercellular cement may change with the respiratory activity of the tissue.

The acid treatment is regarded as operating at the region of the middle lamella, since the cells after treatment are for the most part undamaged. The process is explained conventionally as being the removal of calcium and magnesium ions from the pectate, which allows the polygalacturonide macromolecules to disperse. This explanation describes a simple process of cation exchange followed by the normal consequence of replacing a divalent by a monovalent ion. In terms of this explanation a change in acid lability with anaerobiosis might not be expected. The observed increase in acid-resistant mechanical strength remains unexplained unless in some way as yet undescribed an additional process linked to the respiration of the tissue

is responsible for maintaining it in a state susceptible to acid. In view of the present tendency to regard the intercellular cement as an inert component the effect of anaerobiosis on acid-resistant binding strength appeared to be worthy of quantitative investigation. It may be emphasized that the general topic is one of some importance, since it may be of immediate significance in the interpretation of growth in a tissue.

The design of the investigation has been derived from the character of the original observation. The experimental material has been fragments cut from the apical region of pea roots, and these, after different experimental treatments, have been treated with N. hydrochloric acid under the conditions of the Feulgen procedure. The acid lability of the intercellular cement is determined by measuring the area to which the whole tissue can be compressed under standard conditions, and the results are recorded as the difference between this and the initial area covered by the tissue. A device has been used which applies a force possessing both shear and compression components. The effect of respiratory activity has been examined by incubating the fragments in anaerobic conditions and by treating them with respiratory inhibitors.

Intercellular cohesion has in certain connexions been studied earlier (Ginzberg, 1958; Wood, 1955; McClendon and Somers, 1960). Hitherto, however, there has been no quantitative investigation of the metabolic control of intercellular cohesion.

MATERIAL AND METHODS

Material. Peas (Sutton's var. 'Meteor') were sown in vermiculite in covered pyrex glass dishes and incubated for a period of $2\frac{1}{2}$ days at $26° \pm 0{\cdot}5°$C. At harvest the majority of the seedlings had straight roots $1\frac{1}{2}$ in.–$2\frac{1}{2}$ in. long, with no trace externally of lateral branching. Within this range of size the seedlings were accepted for experimental work after they had been examined to exclude those showing damage, distortion, or abnormal growth.

In the preparatory treatment it was regarded as important that the root tips should reach the incubation vessels as nearly as possible in the physical condition of growth in the vermiculite. To this end the preparation was performed with the minimum of delay during cutting, and the process of storing pea roots in a washing bath before excision was scrupulously avoided.

The root tips, or segments, were cut by hand. Pieces $2{\cdot}5 \pm 0{\cdot}3$ mm. long were used, and although three different techniques were used to obtain the correct size it was found by experience that quick rejection by eye of the grossly erroneous lengths was the greatest safeguard of uniformity. For serial segments, and whenever a batch of only 10 samples was required, a device was used in which a screw of 1 mm. pitch thread was operated by hand and this protruded 10 roots concurrently from a holding brass block. Examination of data obtained in preliminary experiments showed that the variation obtained in the results could not be attributed to the variation in ength of the samples.

Incubation. The root tips were incubated under nitrogen in pyrex glass tubes 2 in. × 1 in. diameter and closed by a rubber bung, which carried the inlet and outlet tubes for the nitrogen treatment. The root tips were placed on a pad of 4 thicknesses of Whatman No. 1 filter-paper trimmed to fit the base of the tube. The pad was moistened with 0·3 ml. of buffer solution prepared by mixing 0·02 M. citric acid with 0·02 M. Na_2HPO_4 in the appropriate quantities to give the required pH. Nitrogen from a gas cylinder was passed as a gentle stream at just sufficient pressure to maintain a head of 1 in. $\pm \frac{1}{2}$ in. of water for the standard period of 2 minutes, and the tube was then sealed and stored at laboratory conditions for the required incubation period. A supply of nitrogen guaranteed 'oxygen-free' was available, and this was used; but it was not found to offer any advantage over commercial-grade nitrogen. On the other hand, control of the amount of fluid added to the filter-paper pad was found to be most important to avoid the alternative dangers of restricting gaseous exchange by using too much fluid, and allowing the tissues to dry out by using too little.

Acid treatment. At the end of the incubation period the 10 replicate root segments were treated with 1 ml. of M. HCl for a standard period of 10 minutes, in a thermostatically-controlled water bath at 60° C.

Test. Essentially the test amounts to 'squashing' the root segment after it has been treated with acid, under conditions chosen to show the relationship between the force applied and the effect on the tissue. There is practical advantage in using a standard 'squashing force' and measuring the resultant increase in area of the tissue, which outweighed in the present work the theoretical advantage of measuring the force required to produce an arbitrarily chosen increase in area.

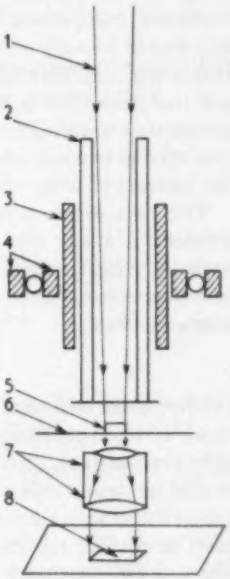

FIG. 1. Diagram of the device used to measure the mechanical strength of root segments.

1. Focused incident light.
2. Glass tube.
3. Copper sleeving tube.
4. Ball-race.
5. Root segment.
6. Microscope glass slide.
7. Microscope eyepiece (× 10 magnification).
8. Cast shadow.

The apparatus is represented diagrammatically in Fig. 1. Light from above passes through the centre tube to focus on the root segment supported on a glass slide. The shadow cast is magnified by a microscope × 10 eyepiece and brought to sharp focus on a sheet of paper below, where it is outlined in ink. A piece of glass tubing approximately 9 cm. long and 7 gm. in weight is held with the lower end (to which a cover-glass is cemented) approximately 5 mm. above the root segment. This forms the standard load for 'squashing' the tissue. The glass tube is held (long axis vertically) in a copper-tube sleeving which is itself mounted (by push-fit) inside a small ballrace, the

outer ring of which is securely clamped in position. On releasing the glass tube from the locked position it can be moved freely in the vertical plane, and can be lowered until it just touches the root segment; it is then allowed to fall freely. The fall of this weight through the tissue can be seen to have a rotational component, thus introducing a shear action, which is further augmented by very gentle rotation by hand of the copper tube. The rotation causes the lock between the glass tube and the copper sleeve to re-engage, and the glass tube is then rotated through a few degrees while still resting on the root tissue. The shadow cast is then outlined in ink again, and subsequently the two outlined shadow-areas are cut out and weighed to determine the increase in area.

The normal practice was to prepare a sample of 10 or more replicate segments for any one determination, but after acid treatment the tissues were very easily damaged by handling. Such damaged specimens were of course rejected, but in no case was the recorded value the mean of less than seven replicates.

EXPERIMENTAL RESULTS

The values recorded are the absolute increases in area of the tissues as a result of the 'squashing' treatment. In each case the value given is the mean of several samples, usually 10 and never less than 7. A tissue highly susceptible to acid treatment will show a low residual mechanical strength, and therefore a large increase in area on testing. Within the limits of size of the root segments used herein, the results showed no significant correlation between the initial size and the increment on 'squashing', and it was concluded from this that an appropriate weight for 'squashing' segments of this size had been chosen.

The random error in the results among any given batch of replicates was quite large, giving rise to standard deviations of 50 per cent. of the mean value for tissues highly susceptible to acid, where the scatter is greatest; but two factors when taken into account combine to make this order of error acceptable. First, in taking readings of mechanical strength the biological variation is always fairly great. Secondly, manipulation is not easily adjusted to particles of $2{\cdot}5 \times 1$ mm. size; e.g. during the incubation it is difficult to ensure that the identical micro-environment exists throughout the batch of samples. Furthermore, there are reasons for believing that the random error should fall as the acid-resistant strength increases, as was in fact observed.

In a systematic search for factors that might be acting to affect either the performance of the test, or the behaviour of the phenomenon under investigation, it became apparent that control of pH during the incubation was important. Variations in the osmotic pressure of the incubation medium were of negligible effect; even the additions of 0·4 M. and 0·6 M. solutions of mannitol produced increases in the acid-resistant mechanical strength that were barely detectable.

In Table I are tabulated the results of an experiment where 2·5 mm.-long

root tips were incubated under air at varied pH for varied times. The results show only a slight linear drift towards an increase in acid-resistant mechanical strength during a period of 8 hours, and no significance in the pH of the incubation fluid within the range pH 2·8–6·7.

TABLE I

The increase in area of 2·5 mm. long root tips caused by 'squashing' them following an incubation in air, during which pH and time were variables

Figures are the increase in area in sq. mm. Each value is the mean from 7–10 replicate samples: value at zero time = 3·85

Incubation time in hours	0·5	1·3	2·5	5·1	8·0
pH 2·8	3·49	3·01	3·01	3·15	2·11
pH 3·9	3·19	2·65	2·87	2·51	2·57
pH 4·7	2·24	2·71	2·37	1·93	1·98
pH 5·5	3·45	2·98	2·36	2·32	2·64
pH 6·7	2·48	2·70	3·02	2·41	2·19

TABLE II

The increase in area of 2·5 mm. long root tips caused by 'squashing' them following an incubation under nitrogen, during which pH and time were variables

Figures are the increase in area in sq. mm. Each value is the mean from 7–10 replicate samples: value at zero time = 3·38

Incubation time in hours	0·25	0·51	1·0	2·0	4·5
pH 2·8	1·85	2·54	2·57	2·40	2·00
pH 3·9	1·76	1·65	2·54	2·12	1·89
pH 4·7	2·22	1·49	2·22	2·19	1·58
pH 5·5	2·19	1·37	2·40	2·27	2·07
pH 6·7	2·03	0·84	1·91	1·71	0·91
pH 7·6	1·11	0·78	1·32	1·05	0·60

In Table II are tabulated the results of a parallel experiment incorporating incubation under nitrogen. The results show an entirely different type of response in this case, and this is shown for the pH values 3·9 and 6·7 in Fig. 2. The tissues show a very marked increase in acid-resistant mechanical strength, which takes place by a process that can be divided into three stages, and which shows a pronounced effect of pH. The initial and the final stage are both fast changes, showing a logarithmic time course, and a rate of change that increases with pH over the range pH 2·8–7·6. Further extension of the range to strongly alkaline solutions (pH 11) has shown that this trend to increased acid-resistant mechanical strength continues well beyond the range of physiological pH.

The initial stage lasts for about 30 minutes and then gives place to the second stage, which is subject to a biological variation which is not yet fully understood. In the cases illustrated in Fig. 2 the second stage lasts for about 30 minutes, during which time the initial tendency is partly reversed. In

other batches the duration of this stage has been longer, lasting up to 1½ hours, and again in some batches there has been only a cessation of the initial process and not a recovery from it. To some extent this variation from batch to batch in response to identical treatment can be correlated with the rate of growth of the peas before harvesting. In the cases where a partial recovery of the initial trend occurs, however, the conclusion seems inescapable

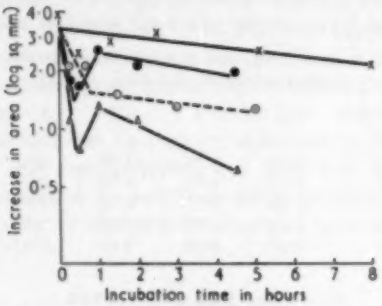

FIG. 2. Increase in area on squashing acid-treated root segments plotted against the time of incubation, illustrating the increase in acid-resistant mechanical strength.

–×—×– Aerobic incubation at pH 6·7
–●—●– Anaerobic incubation at pH 3·9
–⊙– –⊙– Aerobic incubation at pH 6·7 in NaCN concentration 2×10^{-3} M.
–Δ—Δ– Anaerobic incubation at pH 7·6

that a change in the metabolic processes has occurred coincidental with the commencement of the second stage.

The final stage, although continuing the general trend of the initial stage, proceeds at a slower pace, but shows very clearly the effect of pH. Results are replotted in Fig. 3 to show increases in area of tested samples as a function of pH, at the end of the initial and second stages, and the final figures after 4½ hours under nitrogen. The values fit reasonably well to straight lines (the final value at pH 5·5 is considered to be erroneous in this experiment).

The addition of respiratory inhibitors to the incubation fluid of tissues stored under air results in an increase in acid-resistant mechanical strength similar to the effect of incubation under nitrogen. This is illustrated by the effect of cyanide at a concentration of 2×10^{-3} M. at pH 6·7, as the broken line in Fig. 2 shows.

In Table III are tabulated the results of an experiment in which, in 10-fold replication, 6 serial segments of 2·5 mm. length moistened by a solution buffered to pH 6·7 were incubated under nitrogen for variable periods of time up to 4 hours. The results are presented as a histogram in Fig. 4 to show, at the given time periods, the distribution of acid-resistant strength along the root. It is apparent from these data that the segments that are most

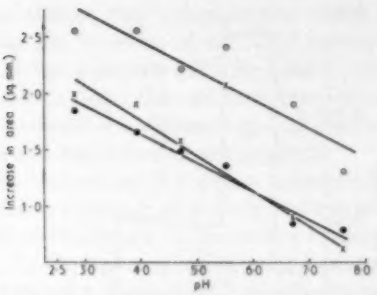

FIG. 3. Increase in area on squashing acid-treated root segments following an incubation under nitrogen, plotted against the pH of the incubation medium (M/50 citrate, phosphate buffer).

–●—●– close of initial stage (approx. 0·5 hrs. incubation)
–⊙—⊙– close of second stage (approx. 1·0 hrs. incubation)
–×—×– final values (4·5 hrs. incubation)

TABLE III

The increase in area caused by 'squashing' 2·5 mm. long serial segments of pea roots, following an incubation under nitrogen at pH 6·7 for variable time periods

Figures are the increase in area in sq. mm. for the mean value of 7–10 replicate samples

Incubation time in hours	zero	0·3	0·5	1·0	2·5	4·6
Position of segment on root in mm.						
0–2·5	2·49	0·86	0·94	1·32	0·48	0·32
2·5–5·0	4·72	1·48	1·24	1·36	0·83	0·65
5·0–7·5	2·95	1·63	0·98	0·93	0·58	0·92
7·5–10·0	3·01	1·09	1·08	0·87	..	0·44
10·0–12·5	1·22	0·76	0·76	0·46	..	0·46
12·5–15·0	1·01	0·63	0·93	0·54	..	0·66

actively engaged in growth, whether by cell division or by cell extension, are the most susceptible to treatment with acid, and also show the most pronounced increase in acid-resistant mechanical strength after incubation under nitrogen for short time periods.

DISCUSSION

In these experiments it was desirable that the respiratory processes were suspended for a period of time without causing permanent respiratory failure, and so the choice fell to the use of periods of anaerobiosis by storage under nitrogen in preference to the use of respiratory inhibitors. Exploratory

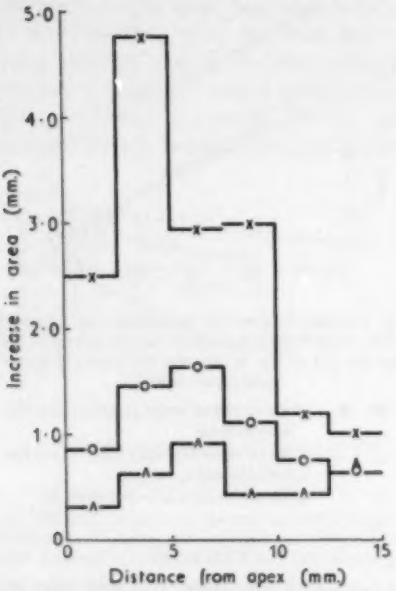

FIG. 4. Increase in area on squashing acid-treated root segments 2·5 mm. long, plotted against the original location of the segment in the intact root.

Incubation at pH 6·7 under nitrogen for the time periods shown.

–×—×– Fresh cut segments.
–o—o– 20 min. incubation.
–∧—∧– 4·6 hrs. incubation.

experiments were conducted, however, in which root tips moistened by solutions of inhibitors were incubated under air; the result of one of these, using sodium cyanide at a concentration of 2×10^{-3} M., is incorporated in Fig. 2. An increase in acid-resistant mechanical tissue-strength is observed, as in the case of nitrogen-treated tissues, and it is clear from these experiments that the condition for promoting increased acid-resistance is an impairment of respiration, which may be either temporary or permanent. In another exploratory experiment di-nitrophenol solution at a concentration of 10^{-3} M. was added to an incubation under nitrogen, producing a result identical with that for nitrogen-treated controls at the same pH.

Fresh untreated root tips have a mechanical strength that is greatly reduced by acid. Preliminary experiments showed that the change is brought about by M. HCl at 60° C. in as short a time as 1–2 minutes, and the succeeding 8 minutes of the treatment produce very little change in the residual mechanical strength. It can also be shown by employing the Feulgen staining technique that the acid is able to penetrate to the nuclei of the cells, and bring

about in them the change that subsequently causes development of the coloration indicating the presence of DNA. It seems reasonable, then, to assume that the acid can penetrate fully the intercellular regions during the treatment, and further that the acid-resistant mechanical strength is dependent on the presence of a different type of chemical linkage from that which gives rise to acid-labile mechanical strength.

The results of the tests on serial 2·5-mm. segments of the root show that the wall of the cell in the process of growth and extension must go through a remarkable sequence of changes in the nature of the linkages that exist in the middle lamella. In the region of cell division both types of linkages occur and the mechanical strength is susceptible to acid treatment. In the region of extension growth the tissues show a very high acid susceptibility, indicating a fall in the number of acid-resistant linkages, and the situation then changes again to a low acid susceptibility, and hence an increase in the number of acid-resistant linkages, when extension is complete. It would be very surprising with such a distribution if there were not a close relationship between the high susceptibility to acid and the need for a type of linkage in the middle lamella that could accommodate the stresses of growth; indeed, although we have no evidence of this, it seems likely that under certain natural conditions the acid-labile linkage is a weaker structure mechanically than is the acid-resistant linkage.

It is noticeable that where the acid susceptibility is high the effect of interrupting the respiratory processes is also pronounced, leading in a very short time to a mechanical strength that is almost the equal of acid-resistant tissues. It is difficult to see how, in the absence of normal cellular activity, this change could be brought about by enzymes providing metabolic intermediates to form linkages across the planes of weakness, except in the case in which the 'bridging molecules' are accumulated as a by-product of anaerobic activity, e.g. CO_2 bridges. An alternative explanation is that the acid-labile linkage is directly converted to an acid-resistant linkage by some change in its environment that is produced by anaerobiosis. If an explanation of this sort is a true description of the state of the tissue, then the total number of linkages changes little (if at all) during the time of cell extension, and the acid-labile linkage stands in some relatively simple relationship to the acid-resistant linkage.

The change that is induced by anaerobiosis is remarkable in several ways. Processes that occur on stopping respiration are typically degradative, involving the breakdown of complex structures and systems, or for example, at the molecular level of complexity, hydrolytic cleavage of large molecules, whereas here the reverse seems to be taking place, i.e. a constructive or synthetic process. The change takes place most readily in alkaline solution, and this is not typical of enzymic processes. The form of the rate-limiting step is a logarithmic time curve, indicating that this is a self-catalysed reaction. These points suggest not a physiological process, but a chemical process (or chain of processes) which is driven in the normal way by a fall

in the chemical energy of the system. In this a limited analogy with the hardening of the insect cuticle following ecdysis can be drawn.

The observations reported in this paper have been discussed at the molecular level, in considering the chemical changes that must be occurring in the middle lamella; at the structural level, in considering the physiological control of the forces that are exerted in the tissue; and at the metabolic level, in considering the known biochemical pathways that could influence or bring about the changes observed. It is clear that much more work must be done before the position is understood, since at none of these three levels is there yet any evidence that would link the observations to our knowledge of cellular processes. It must be accepted, however, that in young root tissues the middle lamella of the cell wall exists in a dynamic state under the control of the general metabolism of the tissue and cannot safely be considered as an inert intercellular cement.

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The Structure and Growth of Root Hairs

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SUMMARY

The walls of normal root hairs of White Mustard (*Sinapis alba*) have been examined by the methods of polarization microscopy, X-ray diffraction analysis, and electron microscopy, and hairs have also been examined after growth in media containing ^{14}C labelled glucose. Autoradiographs show that rapid wall synthesis occurs over a length of about 120 μ back from the tip. The microfibrillar material deposited is Cellulose I, and over the hemispherical tip of the hair the microfibrils are arranged at random. The lamella carrying these microfibrils, which passes back as the outermost lamella of the hair lower down, is classified as an α layer. Within this, reaching almost to the tip, the inner wall lamella has microfibrils running approximately longitudinally. Both these lamellae are incrusted, the outer lamella much more intensely. On plasmoptysis the material ejected from the tip of the hair can be seen after the appropriate treatment to contain microfibrils. It is suggested that the extreme tip of the hair is the site of cellulose synthesis and rapid growth in wall area; and that growth here may be by intussusception. In the region lower down, where the inner lamella is also present, wall deposition is apparently by apposition. This structure appears to lend no support to a simple multi-net growth hypothesis.

INTRODUCTION

In most plants some of the epidermal cells of the root develop tubular outgrowths called root hairs. These have been subjects of discussion ever since they were first described by Malpighi (1686) and Grew (1685) and have long been considered ideal for studies of cell growth. It was realized long ago that growth in root hairs is confined to the tip of the cells (Reinhardt, 1892; Haberlandt, 1914; Windel, 1916; and Guttenburg, 1940). This was demonstrated by studying the relative movements of particles adhering to the wall. There is now complete agreement that the synthesis of cell-wall material leading to increase in wall area is limited to a relatively short portion of the hair behind the hemispherical apex.

Opinion concerning the chemical composition and structure of the root-hair cell wall is, however, by no means so unanimous. Roberts (1916), working with the root hairs of a number of common crop plants, reported that the root-hair wall is continuous with the walls of the hair-forming cell, and that it is made up of two parts, an inner layer of cellulose and an outer layer of calcium pectate. Other workers (e.g. Ziegenspek, 1920) agreed with the general conclusions of Roberts (1916), although they suggested the presence

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of a substance called 'amyloid' in place of cellulose at the tips of the hairs. McCoy (1932), on the other hand, in the course of an investigation into the entry of nodule-forming bacteria in legumes, concluded that the pectic and cellulose constituents of the cell wall are associated together and not separated into different layers. Some of the more recent workers (e.g. Cormack, 1955) support completely the view of Roberts (1916), while others (e.g. Ekdahl, 1957*a* and Stocking, 1956) agree with McCoy (1932) that the pectic and cellulosic substances are uniformly distributed within the hair wall.

As there has been little agreement on the detailed structure of the root-hair wall, it is not surprising that several conflicting theories concerning its growth have been proposed. Cormack (1935, 1937, 1944, 1949, 1955) has presented one of the most detailed hypotheses. He assumes that the pectic substances in the outer layer of the hair wall determine the physical properties of the growing wall and, in particular, that the formation of calcium pectate limits the initiation and growth of the hairs. The root hairs are considered to be produced by internal pressure on the plastic portions of an unequally hardened epidermal cell wall. In the young hair, this softer area is confined to a narrow zone at the tip of the cell, with the result that, so long as calcium is readily available, the growing hairs become long, straight, and narrow. When calcium is not available the softer area becomes greatly extended, and the growing hairs become inflated and branched.

While Cormack's theory has gained considerable acceptance, there are some features which indicate that factors other than calcification are involved. Perhaps the most important of these is the well-known fact that the root hairs of nearly all plants will grow quite satisfactorily in moist air. It is then difficult to support Cormack's theory of gradual calcification of the outer pectic layer in the absence of any external calcium supply. With this in mind Ekdahl (1948, 1953, 1957*a*, 1957*b*) has put forward the view that calcification does not occur, and that hardening is due entirely to changes in the cellulosic substances which involve crystallization of cellulose and the gradual deposition of cellulose strands from the extreme apical point over the cylindrical portion of the hair. The basic difference between the theories of Cormack (1955) and Ekdahl (1957*a*) is to be found in their differing views of the structure of the mature wall.

The first direct observation on root-hair structure by means of the electron microscope was carried out by Frey-Wyssling (1949, 1952). After chemical pre-treatment it was found that the tip of the root hair was covered by a felt-like layer of cellulosic microfibrils. Roelofsen and Houwink (1953), however, using a milder pre-treatment, found no evidence to support Frey-Wyssling's conclusions. The microfibrils in the outer layer were found by them to be oriented at random in a network completely enveloping the tip, with no protrusion such as Frey-Wyssling had described. Roelofsen also described an inner layer of axially oriented microfibrils which he termed the secondary wall. Polarization microscopy showed that this layer extends from the mature wall to a region just below the tip of the hair.

As the result of their investigations of root hairs and other tubular cells Houwink and Roelofsen (1954) have put forward a new concept of growth, based on a much older idea, the *multi-net growth* hypothesis. The relevance of this hypothesis to modern investigations of growing cells and to modern concepts of the growth process has been discussed elsewhere (Preston, 1958*a*, 1958*b*, 1959). The hypothesis involves three main assumptions: (1) all cell-wall lamellae are extending axially until fixation; (2) new microfibrils are continually deposited on to the inner face of the cell wall, so that every layer of microfibrils is gradually moving outwards; and (3) as a lamella is extended axially the constituent microfibrils, which originally tend to lie transversely, become reoriented to a more random or even axial arrangement. The hypothesis demands therefore that, in growing walls of elongated cells, the microfibrils of the innermost wall lamella shall tend towards a transverse orientation, that those of the outer lamellae shall be oriented more at random or even axially, and that there shall be a gradual transition in orientation in intermediate lamellae.

The present work was undertaken in an attempt to elucidate further both the structure of the growing wall in root hairs and its interpretation in terms of this growth mechanism. The investigations of such workers as Cormack and Ekdahl are still based on the concepts of root-hair structure arrived at by the early microchemical staining tests. Recent work on the fine structure of root hairs by Frey-Wyssling and Roelofsen, on the other hand, takes little account of the work of earlier investigators, and is concerned solely with the arrangement of the cellulosic microfibrils to the exclusion of other wall constituents. This is intended to be the first of a series of papers in which the two points of view may eventually be fused.

MATERIAL AND METHODS

Roots of White Mustard (*Sinapis alba*) were grown in a nutrient solution under standard conditions. The seeds, all taken from the same batch, were germinated at 20° C. in petri-dishes lined with filter-paper moistened with glass-distilled water. After 24 hours, seedlings having roots 2 mm.–3 mm. in length were transferred to a culture chamber. This took the form of a rectangular glass tank holding approximately 1 l. nutrient solution. The chamber was filled to the brim with nutrient solution and a 'Perspex' lid fitted so as to exclude any air between the lid and the surface of the solution. Seedlings were placed in rows along 6 bevelled slits running across the width of the lid with their roots passing in the solution below. Approximately 100 roots could be accommodated in the chamber, and provision was made for continuous aeration by means of a rotary air pump. The nutrient solution consisted of potassium hydrogen phosphate (1·0 mM./l.), magnesium sulphate (1·0 mM./l.), and calcium nitrate (2·0 mM./l.), dissolved in glass-distilled water. After transfer to the culture chamber, the temperature of which was maintained at 20°C. $\pm$ 0·5° C., well-developed root hairs were available after 48–60 hours.

Root hairs were harvested by one of two methods. In the simpler method, the hairs were cut from the roots by means of a razor blade and collected by centrifugation. For the electron microscopy of whole hairs, however, particularly of hair tips, a method less likely to cause mechanical disturbances was devised. The roots were fixed in 5 per cent. formalin and washed in water after an appropriate time. They were placed on a Philips grid, with the usual 'formvar' supporting film, in such a way that the tips of a few root hairs lay over the slit. Root hairs were chosen from the region of the root in which the hairs were certainly growing. After drying down, the root was removed and the hair shadowed with Pd/Au. Hairs were also examined electron microscopically after grinding in a Potter homogenizer, and for this purpose hairs were used severed from fixed roots.

In preparations used for ultra-thin sectioning, roots were fixed for 1–2 hours in the normal way in a 2 per cent. osmium tetroxide solution buffered to pH 7·3, washed in dilute buffer solution, taken through the alcohols, and embedded in a mixture of three parts polybutyl to one part polymethyl methacrylate. When necessary, the methacrylate was removed from the sections as described by Myers *et al.* (1956), and the sections were shadowed with Pd/Au. In the chemical pre-treatment of root hairs the methods both of Roelofsen and Houwink (1953) and of Jermyn and Isherwood (1956), as modified by Cronshaw, Myers, and Preston (1958), were followed. The treatments were applied to material fixed in formalin. In the former, the material is first heated to 100° C. for 30 min. in a 1:1 (by volume) mixture of hydrogen peroxide (20 vol.) and glacial acetic acid, then for 20 min. in 2 per cent. (by volume) sulphuric acid, and finally for a further 20 min. in 2 per cent. (by weight) potassium hydroxide. The latter treatment also consists of three steps but mineral acid is avoided. The material is first immersed in distilled water over a boiling-water bath for 12 hours, then treated for 4 hours at 25° C. in 4N. potassium hydroxide, and finally for 4 hours at 75–80° C. in 160 ml. of water containing 1·5 gm. sodium chlorite and 10 drops of glacial acetic acid.

The equipment available at the time did not allow the examination of single hairs by X-ray diffraction analysis. Consequently, powder diagrams were obtained from a pellet of hairs, cut from 150 roots without previous fixation. The pellet was mounted at a distance of 3 cm. from the X-ray film (subsequently calibrated from a silver diagram) and CuKα radiation was used collimated to a beam 0·5 mm. diameter.

Finally, an attempt was made to confirm the restriction of extension growth to the apex by autoradiography of root hairs, using ^{14}C, since the methods previously used were invariably such as to cause some mechanical injury, leading to a temporary cessation of growth. Roots were cultured in a flowing nutrient solution to which unlabelled glucose had been added to the extent of 0·0115 per cent. When the roots were well established, the solution was replaced by a similar one in which the glucose was generally labelled with an activity of 67·5 μC./mg. Roots were removed at intervals, fixed in 5 per cent. formalin, thoroughly washed, and extracted for 12 hours in boiling water.

Autoradiographs were prepared using Kodak AR. 10 fine-grain autoradiographic stripping plates with an exposure time of 3 months.

RESULTS

It will be well to consider first the location of rapid polysaccharide synthesis as determined by autoradiography. An autoradiograph showing several root hairs labelled with ^{14}C as described above, is presented in Plate I, Fig. 1. Cellulose deposition is not uniform but appears to be confined to the tips, extending over a length of approximately 120μ from the apex. This differs from the traditional concept that growth is limited to the hemispherical tip itself in that rapid synthesis appears to take place over some distance down the walls of the hair away from the tip. Over this region the cell contents differ markedly from those found lower down in the non-extending regions. This difference was observed during an investigation made for other purposes. It was decided at the outset of this inquiry that the most promising line of attack on the problem of the growth mechanism of root hairs was to be found in an attempted correlation of various growth phenomena with fine structure. In order to record growth photographically it was decided to utilize the technique of time-lapse cinephotomicrography. The equipment was designed and constructed in this department (Belford, 1958) and will be fully described elsewhere. A small growth chamber was devised to culture a single root in a flowing nutrient solution, using the microscope with the optical tube in the horizontal position. Using root hairs developing in the standard nutrient solution, frames were exposed at intervals of 15 sec. which, when projected at 16 frames per sec., speeds up motion by a factor of 240. On projection these films present a vivid impression of the growing root hair, the violent protoplasmic motion being particularly striking. Although this instrument was put into service too late to be of use in the present investigation in the manner intended, one observation is worthy of record. The protoplast at the tip of the hair invariably differed from that of the body of the hair in a complete lack of vacuolation at the extreme tip, the dense protoplasm tapering away down the sides of the hair as shown in Plate I, Fig. 2.

The X-ray powder diagram of a pellet of root hairs is illustrated in Plate II, Fig. 3. The corresponding spacings show that the crystalline material present is Cellulose I. The considerable amorphous scatter seen in the diagram is largely due to non-crystalline wall components, including hemicelluloses, pectins, and protoplasmic material. The diagram refers, of course, to the mature wall and does not necessarily yield information concerning the wall at the growing tip. Observation of whole cells under the polarizing microscope shows that over the greater portion of a hair much of the crystalline material is specifically oriented. The (double) wall is positively birefringent (i.e. the m.e.p. lies parallel to cell length) except at the extreme apical end, so that over this region some of the molecular chains of cellulose must run axially. The tip itself is almost anisotropic, suggesting that here the chains are oriented at random.

As seen in the electron microscope, the outer surface of the wall of untreated root hairs is lacking in detail (a typical example is shown in Plate II, Fig. 4). After grinding in a Potter homogenizer, this surface presents the same amorphous appearance (Plate III, Fig. 5) but the development of diagonal cracks enables the inner layer of the opposite wall to be seen. This inner surface presents an entirely different appearance; axially oriented microfibrils are clearly visible and amorphous material is almost absent. The occurrence of these axially oriented microfibrils is in complete agreement with the observation of positive birefringence.

Ultra-thin sections of material embedded without previous chemical treatment reveal much protoplasmic detail, but the cell wall appears almost homogeneous. Plate III, Fig. 6 is an electron micrograph of an oblique transverse section through the epidermal cells of the root. The section passes through the base of a root hair, and the cytoplasm lining the wall and the large central vacuole are clearly visible. Removal of the embedding material and subsequent shadowcasting of the specimens leads to the appearance of much more detail in the wall (Plate IV, Fig. 7). The inner layer of oriented microfibrils and the outer amorphous layer are now clearly visible. The former shows some detail and gives the general impression of the presence of microfibrils densely covered in amorphous material. Some disturbance of the oriented layer of microfibrils undoubtedly takes place during the dissolving process, reducing to some extent the degree of orientation. Further observations made on transverse and longitudinal sections of the wall at higher magnifications fully confirmed these observations. A longitudinal section of the wall is illustrated in Plate IV, Fig. 8, showing the microfibrils in side view on the inner surface of the wall.

Removal of the incrusting materials by the method of Roelofsen and Houwink (1953) naturally causes the microfibrils to be more clearly defined. The inner surface of a root hair revealed by grinding (Plate V, Fig. 9) shows well-oriented microfibrils as before, but the outer layer is now clearly revealed to be made up of randomly arranged microfibrils, confirming the observations of Houwink and Roelofsen (1954). Roelofsen and Houwink's extraction method cannot, however, be used in the present whole-mount technique for the isolation of root-hair tips since the whole root disintegrates. For further examination of the tips, therefore, the milder extraction process of Jermyn and Isherwood (1956) was used as modified by Cronshaw, Myers, and Preston (1958). The root hairs then remained attached to the root throughout extraction, retained their shape without crumpling, and the root itself did not disintegrate if handled carefully. An electron micrograph of a whole root-hair tip after this treatment is presented in Plate V, Fig. 10. The outer layer of random microfibrils clearly forms a continuous layer over the hemispherical tip. It is important to note here that the inner, axially oriented microfibrils approach the tip very closely and certainly occur in the region which autoradiographic studies suggest is extending.

Numerous investigators have noted that actively growing root hairs burst,

or undergo plasmoptysis, when subjected to sudden changes in the external conditions, as, for example, when transferred to solutions of low oxygen content or to hypotonic sodium chloride solutions (Ekdahl, 1948; Kopp, 1948; Stier, 1903). Since the hairs invariably burst at the extreme tip, ejecting part of the protoplasm, it was decided to examine such burst hairs in the electron microscope since, if root hairs grow only at the tip, then the site of microfibril synthesis might be ejected with the protoplasm. Entire roots bearing actively growing root hairs were transferred from the standard nutrient solution to 0·075 M. sodium chloride, a solution found to be most effective in causing plasmoptysis. After washing in distilled water the hairs were placed on electron microscope grids by the whole-mount method and shadowed. The cell contents bursting from the tip of the root hair proved too electron dense for the observation of any detail and accordingly a mild pre-treatment was carried out before examination. Water-soluble material was removed by extracting with water for 12 hours over a boiling-water bath, and much of the protoplasmic material was removed by subsequent treatment with a 0·1 per cent. (by weight) aqueous solution of trypsin at pH. 8·0 and 30° C. for 24 hours. Much of the material ejected from the root hair was then seen to be microfibrillar (Plate VI, Fig. 11). The microfibrils appear to be intertwined and apparently anchored in the electron-dense material at the tip of the burst hair.

DISCUSSION

The wall of root hairs thus consists of two layers which are clearly distinct. The outer surface of chemically untreated hairs appears completely amorphous with no sign of any microfibrillar organization (Fig. 3). Little or no amorphous wall material appears to be associated with the inner surface of the wall where axially oriented microfibrils are clearly visible without chemical pre-treatment. This distinction between the outer and inner layers is equally evident in sections (Fig. 5), and these reveal a well-defined discontinuity between the layers with no suggestion of a gradual merging of one into the other. When incrusting substances have been removed, however, random microfibrils are clearly visible in the outer layer (Fig. 10) continuous over the root hair including the hemispherical tip. This layer of randomly arranged microfibrils is thus normally associated with amorphous wall constituents. The electron micrographs of chemically pre-treated root-hair cell walls showing dense, closely interwoven microfibrils do not, therefore, give a true picture of the state of affairs in the living root hair. In the latter case the cellulosic microfibrils undoubtedly form a very loose network associated with highly hydrated amorphous substances such as pectins and hemicelluloses. The initial observations in the electron microscope on root hairs that had not been chemically pre-treated clearly demonstrated the distribution of amorphous constituents within the cell wall, a factor which appears to have been overlooked in recent studies in the cell-wall structure of root hairs (Roelofsen and Houwink, 1953; Frey-Wyssling, 1952).

The two layers of microfibrils in the root-hair wall are fundamentally

different both in appearance, orientation, and in the association with the other wall components, and it is an over-simplification to consider only the microfibrillar architecture of chemically pre-treated root hairs. This fundamental difference between oriented and randomly arranged microfibrils in plant cell walls in general is fully discussed elsewhere (Belford, Myers, and Preston, 1958). These workers have shown that the application of the terms primary wall to wall layers with randomly arranged microfibrils and secondary wall to layers showing orientation, although widespread, is inaccurate in many cases and new terms have been proposed, namely, that randomly arranged microfibrils associated with amorphous wall constituents should be termed an α layer and the oriented microfibrils with little or no encrusting material should be termed a β layer. This terminology will be used throughout the following discussion.

The large measure of disagreement among earlier workers concerning the chemical make-up of the root-hair cell wall appears to lie in the unsatisfactory nature of microchemical staining tests used. For instance, as early as 1935 Bailey and Kerr (1935) demonstrated that the ruthenium-red test for pectins gives a positive result with hydrated and oxy-cellulose. Tests for cellulose and callose can lead to conflicting results (Nicolai and Preston, 1952), as cellulose can be present even though the staining reaction fails and a positive staining reaction can be equally misleading. These microchemical staining tests do not, therefore, possess the requisite sensitivity or specificity and are, at best, but a rough guide to the general nature of the chemical composition of plant cell walls. The crystalline (microfibrillar) component of the root-hair cell walls investigated during the present inquiry is undoubtedly Cellulose I. The amorphous wall material, located in the outer layer, is rather more difficult to identify. Earlier workers in general presumed an association of pectic compounds with cellulose in the root-hair wall. When examined in the electron microscope after water extraction or acid-ammonium oxalate treatment, amorphous wall material still appears to be present. It is probable that the amorphous component of the wall is, in common with other walls of this nature, largely made up of pectins and hemicelluloses with perhaps some protein material. The latter is suggested by the considerable clearing action of trypsin on the outer layer, and the fact that during the modified Jermyn and Isherwood (1956) extraction the outer layer of random fibrils is not completely revealed until the final chlorite treatment.

With regard to the distribution of these wall components it has been seen from the electron micrographs that the α layer of microfibrils, in association with the amorphous wall constituents, forms a continuous layer which reaches completely over the hemispherical tip. The inner β layer with axially oriented microfibrils, in view of the positive birefringence of the wall, probably extends along the length of the root hair to just below the hemispherical tip.

Cormack's (1955) view of the wall as a two-layered structure with an inner layer of cellulose and an outer layer of calcium pectate (replaced by pectin at the tip) thus has some foundation, but to his conception of the outer layer

must be added the random cellulose microfibrils. If, however, as he supposed, the extreme tip contained a high proportion of pectin this would be expected to show an appearance in the electron microscope different from that of the body of the hair after boiling-water extraction. No such difference was noted either with hairs growing until the moment of fixation or with mature hairs. Cellulose microfibrils undoubtedly provide the main structural component of the wall, extending over the entire tip of the hair, and it appears doubtful if Cormack's hypothesis can provide an acceptable mechanism for the control of the growth. The normal growth of root hairs in moist air gives rise to another serious criticism of Cormack's theory. It would appear that calcium is not directly linked to the calcification of pectins in the wall as postulated by Cormack, but exerts its effect through the general metabolism of the cell. Ekdahl (1957), on the other hand, though placing the emphasis on cellulose synthesis, is mistaken in assuming that the pectic and cellulosic substances are distributed uniformly throughout the wall and that the cellulosic fraction as a whole becomes more or less oriented in a direction parallel to the long axis of the root hair. Moreover, it seems unlikely that 'crystallization' of cellulose takes place gradually in passing from the extreme apical point to the cylindrical part of the hair. It has been shown above that the entire tip is covered by a continuous α layer. Both authors are clearly to some extent correct, Cormack in postulating a two-layer wall structure and Ekdahl in placing emphasis on the synthesis of cellulose.

No evidence has been found during the present investigation to support the existence of the felt-like layer of microfibrils at the tip of the root hair reported by Frey-Wyssling and Muhlethaler (1949). Roelofsen and Houwink (1953) arrived at substantially the same conclusions as those reached in the present investigation with regard to the microfibrillar architecture of the root hair and upon them founded the multi-net hypothesis of cell-wall growth (Houwink and Roelofsen, 1954). As stated earlier, this hypothesis attempts to explain the structure of several types of tubular cell by postulating a gradual reorientation. Roelofsen ascribes this to the fact that growth is confined to the hemispherical tip, but it is nevertheless clear that the multi-net hypothesis has no real basis as applied to root hairs. The inner layer of oriented microfibrils is considered by Roelofsen to be the secondary wall deposited in the cylindrical part of the hair. This terminology has been fully discussed elsewhere (Belford, Myers, and Preston, 1958), and it is suggested that the term *secondary* wall should not in fact be used in this connexion.

To consider now the growth mechanism of the root hair in view of the fine structure revealed in this investigation we may first turn to the electron-microscopic study of plasmoptysed root hairs. Since the α layer is continuous over the tip in actively growing root hairs, the microfibrils are presumably displaced from the extreme tip as new microfibrils are deposited from within. In the examination of plasmoptysed root hairs it was hoped that the site of microfibril synthesis would be ejected from the tip during the almost explosive bursting of the root hair. After removing much of the protoplasmic

material by means of boiling-water extractions and treatment with trypsin (Fig. 11) it was found that much of the ejected material was microfibrillar in nature. The general appearance and loose texture of these microfibrils suggest that they had been recently formed at the tip of the root hair and were ejected with the associated protoplasm. In this α layer the amorphous material is probably of great importance, forming a gelatinous matrix into which new microfibrils are incorporated from within the hemispherical tip as a loose intertwined network. It seems reasonable to assume that the synthesis of this α layer may be confined to the hemispherical tip since the inner β layer of oriented microfibrils, in which but little amorphous material occurs, extends along the cylindrical part of the root hair to the hemispherical tip, virtually isolating the α layer from the protoplasm except within the extreme tip portion.

The pattern of cellulose synthesis revealed by the autoradiographic examination of ^{14}C labelled root hairs (Fig. 1) does not at first sight conform to the classical concept that growth is limited to the extreme tip of the root hairs. All of the earlier investigators (Reinhardt, 1892; Haberlandt, 1914; Windel, 1916; Guttenberg, 1940), however, were observing, by means of marking in some way or by staining, the extensions of the *outer* layer of the wall only, whereas autoradiographic examination reveals the location of synthesis of the *entire* cell wall. The observations of the earlier investigators is completely in line with the origin of the α layer as outlined above, while the blackening of the autoradiographic emulsion for a short distance along the cylindrical part may clearly be ascribed to the synthesis and deposition from within of the oriented microfibrils going to make up the β layer.

Further evidence for this concept of cell-wall synthesis may be obtained from a careful examination of the accelerated protoplasmic streaming to be observed when the time-lapse cinephotomicrographic films of normal root-hair growth are projected. The protoplasm within the mature cylindrical part of the hair is vacuolated and shows violent protoplasmic movement and vacuolation. The protoplasm within the hemispherical tip, on the other hand, appears dense and completely non-vacuolate, corresponding to the region of α layer synthesis. This non-vacuolate protoplasm tapers away down the sides of the hair from the tip, over the region of β layer synthesis, until within the mature root-hair wall the protoplasm is completely vacuolate with but a thin peripheral lining of protoplasm.

It is suggested, therefore, that cell-wall material is synthesized at the extreme apex of the root hair, the cellulose microfibrils forming a loose intertwined network in a gelatinous matrix of pectins, hemicelluloses, and other amorphous wall constituents, new wall material being constantly added by 'intussusception' from within, the existing α layer being displaced towards the cylindrical part of the hair. In the region of this cylindrical part of the hair adjoining the tip, the α layer is reinforced by the 'apposition' of the cellulosic β layer from within giving rise to the mature wall structure revealed in the course of this investigation.

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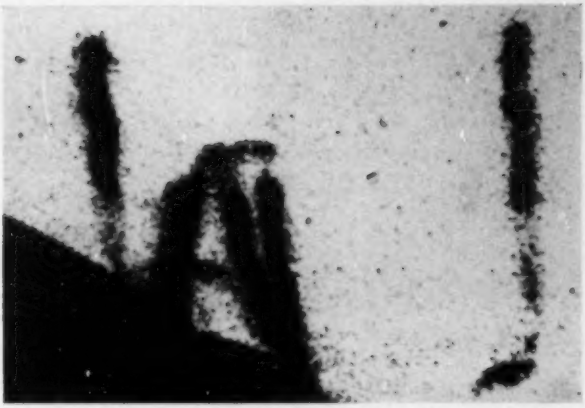

FIG. 1. Autoradiograph of ^{14}C-labelled root hairs. Fixed after 20 min. growth in labelled solution. Magnification × 400.

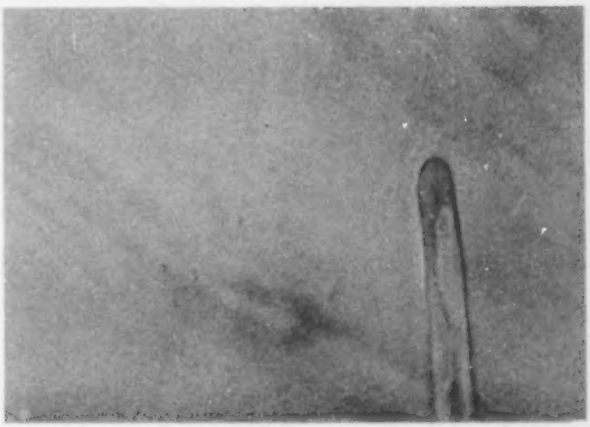

FIG. 2. An enlargement taken from a 16 mm. time-lapse cine film of a growing root hair. Magnification × 500.

D. S. BELFORD AND R. D. PRESTON—PLATE I

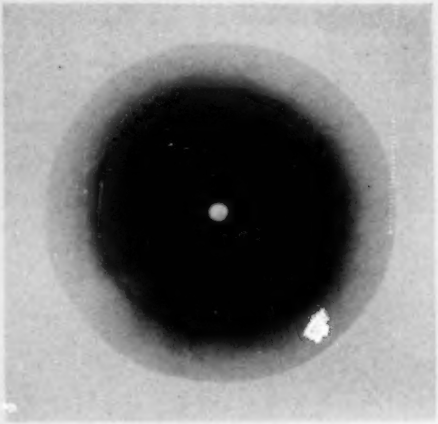

FIG. 3. An X-ray diffraction diagram of a pellet of root-hair cell walls. CuKα radiation.

FIG. 4. Electron micrograph of a whole mount of a root hair. Untreated. Shadowed Pd/Au. Magnification ×6,000.

D. S. BELFORD AND R. D. PRESTON—PLATE II

FIG. 5. Electron micrograph of a root hair after light blending. Untreated. Shadowed Pd/Au. Magnification ×2,975. In the lower portion microfibrils can be seen in the inner lamella of the lower wall. The torn edge on the right is approximately parallel to hair length.

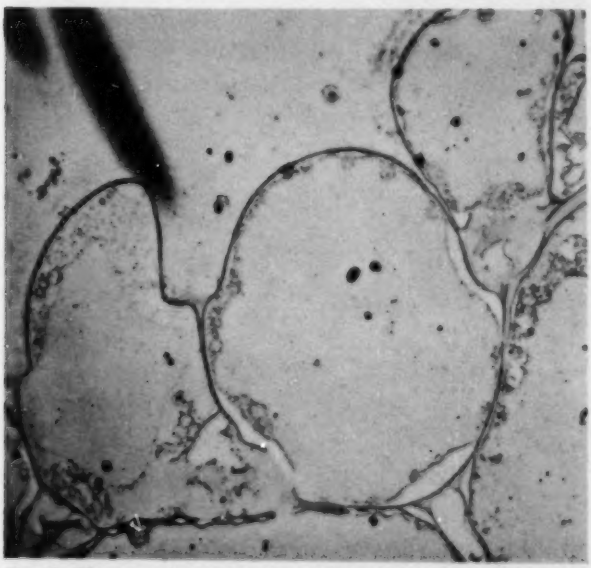

FIG. 6. Electron micrograph of an oblique ultra-thin section of the epidermal cells of the root, passing through the base of a root hair. Untreated. Methacrylate not removed. Unshadowed. Magnification ×2,100.

D. S. BELFORD AND R. D. PRESTON—PLATE III

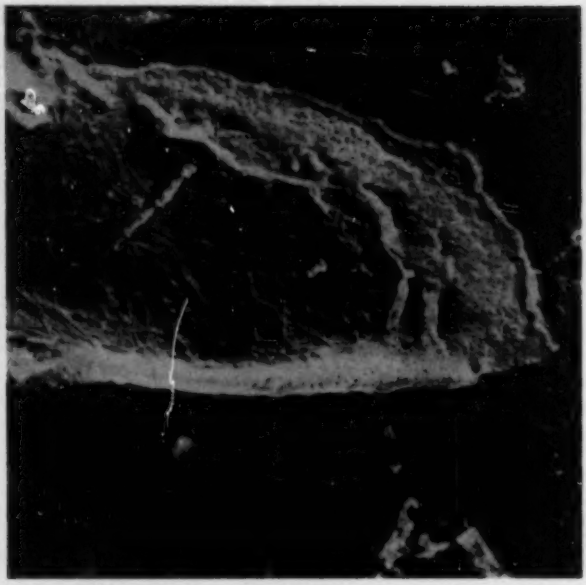

FIG. 7. Electron micrograph of a longitudinal ultra-thin section of a root hair, passing obliquely through the wall. Untreated. Methacrylate removed. Shadowed Pd/Au. Magnification ×22,500.

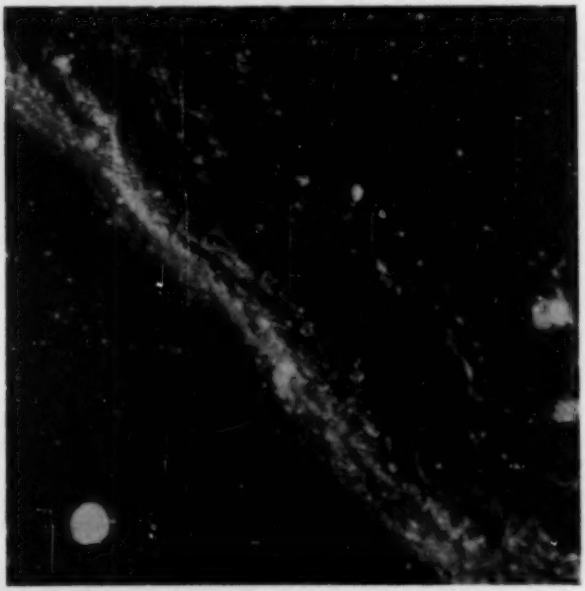

FIG. 8. Electron micrograph of a longitudinal ultra-thin section through the cell wall of a root hair. Untreated. Methacrylate removed. Shadowed Pd/Au. Magnification ×45,000. The outer face of the wall is the lower left face.

D. S. BELFORD AND R. D. PRESTON—PLATE IV

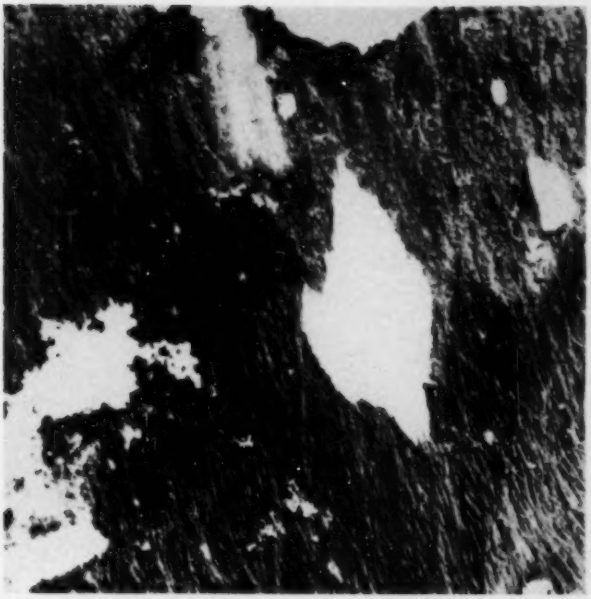

FIG. 9. Electron micrograph of a root hair after light blending. Pre-treatment after Roelofsen and Houwink (1953). Shadowed Pd/Au. Magnification ×15,750.

FIG. 10. Electron micrograph of the tip of a root hair whole-mount. Pre-treatment modified after Jermyn and Isherwood (1956). Shadowed Pd/Au. Magnification ×10,500.

D. S. BELFORD AND R. D. PRESTON—PLATE V

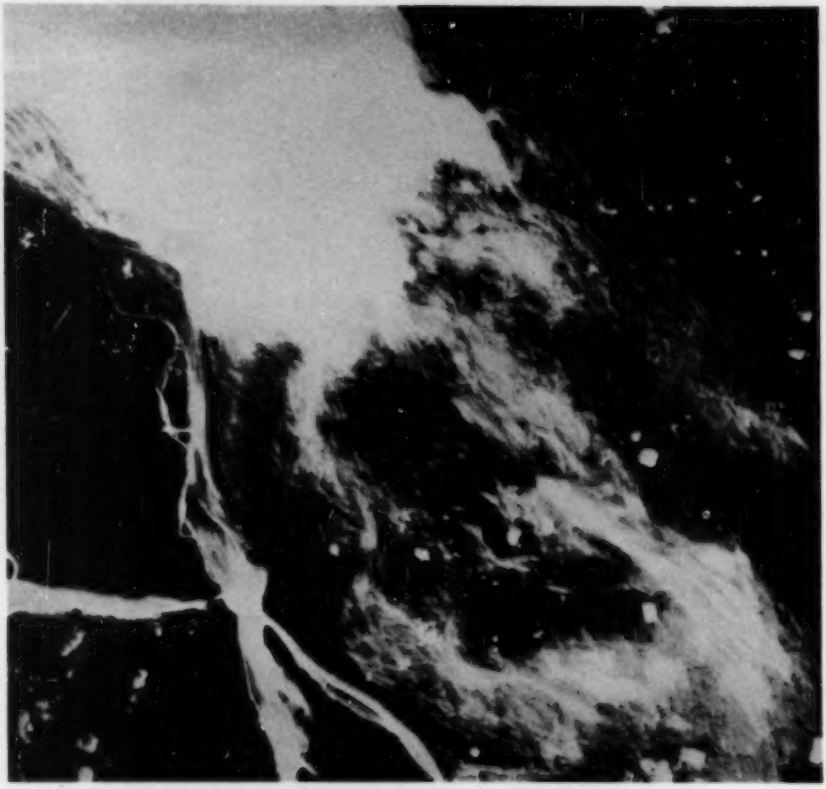

FIG. 11. Electron micrograph of the tip of a root hair after plasmoptysis and treatment. Shadowed Pd/Au. Magnification ×22,500. Note the abundant microfibrils in the exploded material.

D. S. BELFORD AND R. D. PRESTON—PLATE VI

An Enzymic Basis for Pathogenic Specificity in *Ophiobolus graminis*

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With two Figures in the Text

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SUMMARY

Avenacin, the glucosidic inhibitor present in oat roots, is acted on by a specific glucosidase produced by *Ophiobolus graminis* var. *avenae*, which destroys biological activity, as does hydrolysis with 0·1 N. HCl. Neither *O. graminis* itself nor any other fungus tested produces this enzyme. It is suggested that the resistance of oats to *O. graminis* and its susceptibility to var. *avenae* depend on an inhibitor-inactivating enzyme complex, and this is compared with the antibiotic-inactivating enzyme complex found in penicillin-producing moulds and resistant penicillinase-producing bacterial strains; such a complex may be concerned in other cases of pathogenic specificity. The way in which a variety of *O. graminis* pathogenic to oats may have arisen is discussed.

INTRODUCTION

THE inhibitor from oat roots described in previous papers (Turner, 1940*b*, 1952, 1956, 1960) has now been purified by Drs. H. K. Mitchell and J. V. Maizel, and named avenacin (Mitchell and Maizel, 1960). They find that it is identical with the fluorescent 'root tip glucoside' described as present in meristems of oat roots by Goodwin and co-workers (Goodwin and Kavanagh, 1948; Goodwin and Pollock, 1954). They report that the concentration reducing growth of *Neurospora crassa* to 50 per cent. of the control is 0·5 μg./ml., and that hydrolysis with 0·1 N. HCl releases 2 molecules of glucose and 2 molecules of a pentose sugar, leaving the aglycon still fluorescent. They kindly supplied some crystalline avenacin with which the investigations into the cause of pathogenic specificity in *Ophiobolus graminis* described below were carried out.

Growth of *O. graminis* is inhibited by an extract from oat roots which supports good growth of *O. graminis* var. *avenae*, whereas crudely purified preparations of the inhibitor from the root extract are almost equally toxic to both varieties (Turner, 1956). This suggests that either some other constituent of oat sap enables var. *avenae* to overcome the effects of the inhibitor, or that this fungus can destroy the inhibitor when growing in the otherwise favourable medium of the extracted sap. The work described below shows that var. *avenae* produces in low yield an enzyme which splits the glucoside and results in its inactivation.

METHODS

The strains of *O. graminis*, *O. graminis* var. *avenae* and *N. crassa* used were those described in the preceding paper, and the nutrient culture medium was that described earlier (Turner, 1956).

Enzymic preparations from the culture media were made according to the method described by Jermyn (1959) for concentrating β-glucosidase from fungal culture media. The fungi were grown in 250 ml. flasks each containing 50 ml. medium. When fungal mycelium covered the surface of the medium, the fungus was filtered off and the medium from three flasks bulked. To 100 ml. filtrate was added 200 ml. ethanol and the flask was stored overnight at 0°C. The precipitate was centrifuged down, taken up in 10 ml. water and dialysed against running water for 24 hours. It was then incubated for 24 hours at 35°C. with 50 μg. avenacin (added as 0·5 ml. of a 100 μg./ml. solution in 80 per cent. ethanol) dried by boiling and the avenacin recovered in 1·5 ml. ethanol. After filtration 0·3 ml. of this was put as a spot on Whatman's 3 MM paper. The chromatogram was run (ascending) in *n*-propanol: 1 per cent. ammonia (3:1) and examined under U/V light to determine the position of the fluorescent spot.

RESULTS

Enzyme concentrates were made from culture filtrates of the following 8 fungi: *Ophiobolis graminis* (W_1), *O. graminis* var. *avenae* (O_2 and O_8), *Neurospora crassa*, *Aspergillus niger*, *Penicillium notatum*, *Gliocladium roseum*, and *Rhizopus nigricans*.

Fig. 1 shows diagrammatically the position on the chromatograms of avenacin at RF 0·74 and of its aglycon (resulting from hydrolysis with 0·1 N. HCl) at RF 0·91. It shows that incubation with the enzyme preparations from O_2 and O_8 culture filtrates has also released the aglycon but that no other culture filtrates were active. Boiling the preparations destroyed activity. Addition of 2 drops of hydrogen peroxide to the O_2 extract reduced activity. A commercial sample of β-glucosidase was not effective when incubated with 50 μg. avenacin in 10 ml. phosphate buffer (Fig. 2).

After incubation with the preparations from W and O_2 culture filtrates avenacin was tested for biological activity against *N. crassa* by the slide assay test described in the preceding paper (Turner, 1960). Table I shows that the O_2 preparation reduced activity, as did hydrolysis with 0·1 N. HCl.

Culture filtrates of O_2 and O_8 thus contain an enzyme which splits the glucoside avenacin, releasing the aglycon and destroying biological activity. Under the conditions of the experiments most of the glucoside was split so that the fluorescent spot at RF 0·91 was brighter than that at 0·74, but some remained unhydrolysed and some biological activity remained in the solution. The effect on the enzyme of hydrogen peroxide is in line with the reported inhibition of β-glucosidases by oxidizing agents (Hestrin *et al.*, 1955). However, the lack of activity of commercial β-glucosidase against avenacin, the inactivity of *P. notatum* and *G. roseum* (both reported by Jermyn (1959)

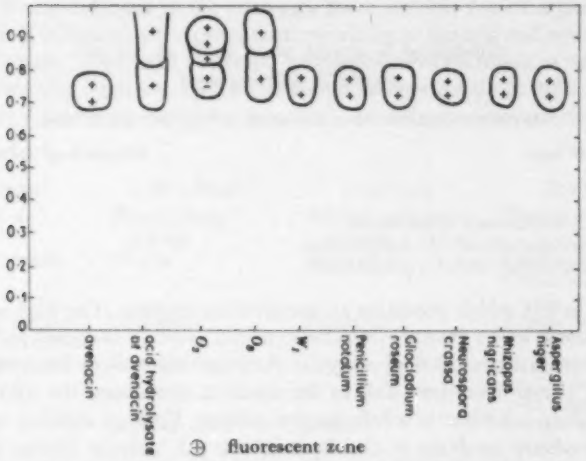

FIG. 1. Chromatogram showing the effect of treatment of avenacin with extracts from culture filtrates of various fungi.

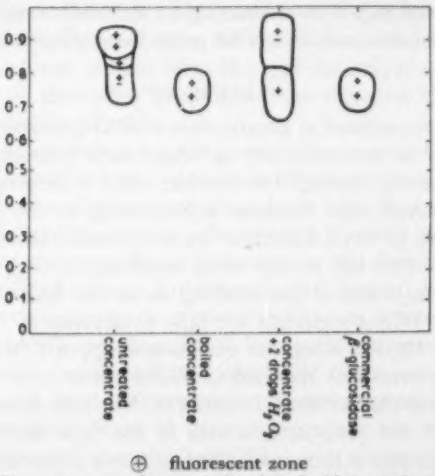

FIG. 2. Chromatogram of avenacin incubated with treated concentrate from culture filtrate of O_3 and with commercial β-glucosidase.

to be active producers of β-glucosidase), and the fact that both W_1 and O_3 culture filtrates showed α-glucosidase activity against maltose, suggest that the avenacin-splitting enzyme is a specific one and it may be termed avenacinase.

With the exception of *O. graminis* and *N. crassa*, the fungi here tested for avenacinase activity are all insensitive to avenacin. Their immunity is clearly not comparable to the condition found in var. *avenae* which is highly sensitive

TABLE I

Growth of N. crassa *in 18 hours on 0·1 ml. streaks of nutrient agar*

Avenacin concentration adjusted to 1 μg/ml. for testing.

Additions to agar	*Diameter of colonies in mm.*
Nil	25
Avenacin	5
0·1 N. HCl hydrolysate of avenacin	25
Avenacin incubated with W_1 preparation	7
Avenacin incubated with O_3 preparation	18

to avenacin but which produces an inactivating enzyme. The high sensitivity of var. *avenae* when grown in synthetic media must be assumed to be due to too slow an accumulation of enzyme. Activity has always been assayed by placing a 3 mm. inoculum disk in the medium containing the inhibitor and not by adding inhibitor to a fully grown culture. Yield or stability of enzyme in the synthetic medium is clearly low, 100 ml. culture filtrate providing insufficient to convert all of the 50 μg. avenacin added. It may be postulated that the yield or stability of enzyme increases in the favourable medium provided by the cell sap, thus accounting for the obvious differential effect of oat root sap on the two varieties. This point has not been investigated.

DISCUSSION

When oats are inoculated at germination with *O. graminis* (W) the seedling roots are invaded in the same way as wheat roots (though less vigorously) with hyphae growing through the cortical cells, ramifying freely; but the infection is transitory, the hyphae disintegrating in the cells and mostly disappearing after 30 days. Infection by var. *avenae*, however, persists and the hyphae grow through to the stele, resulting in brown lesions on the roots and often in death of the seedling as in the take-all stage in wheat (Turner, 1940*a*). Wheat seedlings are fully susceptible to infection by both varieties. In the United Kingdom var. *avenae* appears in widely scattered localities, having been first reported in Wales where it is widespread, then from Scotland and the northern counties of England (Garrett and Dennis, 1943). Because of the resistance of oats to the type variety it is common practice to include oats in the rotation between two wheat crops (e.g. Garrett, 1937). The overwintering mycelium of *O. graminis* in the soil is therefore frequently in a position to cause the transitory infection of oat roots described above, before disintegrating in the cells and being starved out in the surrounding soil where, as a root-inhabiting fungus of low competitive saprophytic ability, its survival is limited (Garrett, 1950). A mutation in an infecting hyphal cell which enables the hyphae to overcome the resistance of the oat root and to develop to full colonization would clearly be of great survival value. Resistance appears to be due to the inhibitor avenacin, so that the production of an enzyme inactivating avenacin would have this effect,

provided that conditions in the root cells were suitable for its activity. Since the source of infection is the persistant mycelium in the soil and not air-borne spores (Garrett, 1939), the scattered geographical occurrence of var. *avenae* may indicate that such a mutation has arisen independently in different areas.

The two fungal varieties differ from each other in the characteristics listed below:

Character	*W isolates*	*O isolates*	*Reference*
Pathogenic to	Wheat, some grasses	Wheat, oats, some grasses	Turner, 1940*a*
Ascospore length	70–90μ	96–123μ	Turner, 1940*a* Garrett and Dennis, 1943
Microconidial production in culture	Occasional	Frequent	Turner, 1940*a*
Spermagonia on host	Not described	Found	Jones, 1926 Smith, personal communication
Colour	Light to dark	Dark	Unpublished observation
Reaction to cystine and cysteine	Growth stimulated	Growth inhibited	Turner, 1957

One must assume, therefore, either that the capacity to cause lasting infection in oats due to production of avenacinase was the fundamental mutation separating var. *avenae* from the type variety, the other characters developing later, or that other conditions were required before production of avenacinase could be effective.

The difference in spore length holds good in the majority of cases examined but there are records of isolates with spores of intermediate length (Bradley-Jones, personal communication) and it has been suggested that this may not be a valid criterion for differentiating the varieties (Garrett and Dennis, 1943). The production of microconidia in spermagonia was described by Jones for *O. graminis* established on oats (presumably var. *avenae*) and confirmed by Smith (1956) for this variety on *Agrostis* spp. Jones found that the microconidia were apparently non-functional. Production of spores is a character known to vary with nutritional and metabolic characters, and O isolates generally lose the capacity after repeated sub-culturing, and do not produce spermagonia in culture. None of these spore characters at present seems relevant to the difference in pathogenicity between the two varieties.

The dark colour of mycelia of some W and all of the O isolates examined is apparently not due to melanin (since strong reducing agents decolorize the mycelia) but to some comparable product of oxidation, which develops only in the presence of air and not in tightly stoppered bijou phials. White (1942) showed that 8 ascospores from a single ascus of *O. graminis* gave rise to 4 light- and 4 dark-coloured colonies, segregation for colour being independent of that for high and low virulence to wheat. Possibly production or quantity of a substrate capable of being oxidized to a black product, or of the oxidizing enzyme, is a gene-controlled character present in some W and all O isolates.

The reaction to cystine and cysteine is the easiest way of distinguishing between the two varieties in the laboratory. Of 6 W and 18 O isolates from different parts of the country all have shown this difference. The isolates with spores of intermediate length, whether obtained from wheat or from oats, all reacted as O isolates; no short-spored isolate reacted as an O isolate. This suggests a metabolic difference between the two varieties and some work has been done on it but with inconclusive results (Turner, 1959). There may be a connexion with a high reductive capacity of O mycelia as indicated by decolorization of indophenol by culture filtrates and mycelial homogenates but the evidence is slight.

The hypothesis may be put forward that certain conditions are necessary for a mutation to avenacinase production in hyphal cells inside an oat root to be effective. Of these, one may be maintenance of the environment of the hyphae in a reduced state so that the glucosidase (apparently inhibited by oxidizing agents) may function. A preliminary mutation to a potentially dark colour, produced by oxidation of a substrate, may therefore be necessary. If such were the case, only dark-coloured W mycelia, invading oat roots, would have a chance of utilizing a mutation to enzyme production.

The production of avenacinase may be compared with the production of penicillinase by strains of *B. cereus* (Pollock, 1953) which would enable these cells to proliferate in the neighbourhood of penicillin-producing moulds in the soil. Oats may be regarded as containing a built-in antibiotic, avenacin, which prevents colonization of the roots by sensitive fungi such as *O. graminis*; a mutation to enzyme production (in this case constitutive), given other favourable conditions, would render the fungus resistant to the antibiotic. The case may be used to illustrate the hypothesis that virulence and specificity are separate problems, and that specificity, not virulence, is in question here. *O. graminis* is capable of invading both hosts, although virulence may be lost after culture outside the plant and it will not then be able to invade either; or certain isolates may be of low virulence (White, 1942). But, whether virulent or not, *O. graminis* does not possess the enzymic property necessary for sustained infection of oats; this is specific to var. *avenae*. It seems likely that the inhibitor-inactivating enzyme complex described here may be encountered in other problems of specificity. Thus Buxton's work on *Fusarium oxysporum f. pisi* (1957), showing inhibition of germination of spores of strains of the fungus by exudates from roots to which they are not pathogenic, while pathogenic strains can germinate in these exudates, may suggest a similar mechanism. Furthermore, findings of Dickson and collaborators concerning the necrotic-fleck reaction of varieties of corn to strains of *Puccinia sorghi*, which they describe as indicating that all strains of the fungus produce a toxin calling forth this reaction but that certain varieties of the host avoid reacting to it (Dickson *et al.*, 1959), suggest the same complex in the reverse direction, i.e. a metabolite produced by the fungus but inactivated by the host plant.

ACKNOWLEDGEMENTS

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